

Estrogen receptor α -351 *Xba*I*G and -397 *Pvu*II*C-related genotypes and alleles are associated with higher susceptibilities of endometriosis and leiomyoma

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Endometriosis and leiomyoma are both common estrogen-related gynaecological diseases. We aimed to elucidate the association of estrogen receptor α (ER α)-351 A>G (*Xba*I) and -397 T>C (*Pvu*II) gene polymorphisms with endometriosis and leiomyoma. Women were divided into three groups: (i) severe endometriosis ($n = 112$), (ii) leiomyoma ($n = 106$) and (iii) normal controls ($n = 110$). Genomic DNA was obtained from peripheral leukocytes. ER α -351 A/G *Xba*I and -397 T/C *Pvu*II polymorphisms were assayed by the method of PCR and restriction fragment length polymorphism (RFLP). Genotypes and allelic frequencies in each group were compared. The genotype/allele frequencies of ER α -351 and -397 polymorphisms in endometriosis or leiomyoma groups were different from those of normal controls. ER α mutant-related genotypes/alleles (-351G and -397C) presented higher percentages in the endometriosis/leiomyoma population compared with normal controls. Proportions of ER α -351 AA/AG/GG genotypes and A/G alleles in each group were (i) 26.8/57.1/16.1 and 55.4/44.6%; (ii) 19.8/52.8/27.4 and 46.2/53.8% and (iii) 33.6/64.6/1.8 and 65.9/34.1%. Proportions of ER α -397 TT/TC/CC genotypes and T/C alleles in each group were (i) 24.1/60.7/15.2 and 54.5/45.5%; (ii) 23.6/70.8/5.6 and 59/41% and (iii) 54.5/40/5.5 and 74.5/25.5%. We concluded that ER α -351 *Xba*I*G- and -397 *Pvu*II*C-related genotypes/alleles were correlated with higher susceptibilities of endometriosis or leiomyoma, which might be associated with related pathogenesises.

Key words: endometriosis/estrogen receptor/leiomyoma/polymorphism/single-nucleotide polymorphism

Introduction

Endometriosis, a common gynaecological disorder in premenopausal women, occurs in around 10% of the female population (Goldman and Cramer, 1989) and as high as 30–40% in infertile women (Strathy *et al.*, 1982). Endometriosis is a polygenic/multifactorial disease, which is related to the complex interactions between hormone and cytokine activation, immunoinflammatory processes, genetic factors and the environment (Kennedy, 1998). Three possible theories have been proposed for endometriosis, including (i) retrograde menstruation through the fallopian tubes into the peritoneal cavity, (ii) lymphatic and vascular metastases and (iii) tissue *in situ* metaplasia. Numerous factors have been implicated in the formation of ectopic endometrium implants, including the ovarian hormones, estrogen and progesterone (Irahara *et al.*, 2001). Estrogen secreted by the ovaries is necessary for the development of endometriosis. Endometriosis develops mostly in women of reproductive age and regresses after menopause or ovariectomy, which suggests estrogen-dependent growth. Ectopic endometrium persistently expresses estrogen receptor (ER), independently of the menstrual phase. Furthermore, the biological activity of the activated receptor in ectopic implants is thought to differ from that in eutopic endometrium (Nisolle *et al.*, 1997).

Leiomyoma, the most common neoplasma of uterus, occurs in around one-fourth of women during their lifetime (Cramer, 1992). Despite its high prevalence, the related pathophysiology and proliferative pathway remains obscure. One possible mechanism is the different expression of estrogen-regulated genes between leiomyoma and normal myometrium (Andersen and Barbieri, 1995). Leiomyoma tissue appears more sensitive to estrogen than myometrium. Myoma growth is regulated not only by serum estrogen but also by estrogen in the tumour itself (Urabe *et al.*, 1990). Tissue concentration of estrogen is higher in leiomyoma tissues than in normal myometrium (Urabe *et al.*, 1990). In addition to sexual hormones, the pathogenesis of leiomyoma involves multiple local growth factors, acting in an autocrine or paracrine fashion (El-Badry *et al.*, 1991). Although the pathogenesis of endometriosis or leiomyoma remains unclear, both gynaecological diseases are known to be estrogen-dependent and have a genetic component. The effects of estrogens are mediated primarily through ER in endometriosis or leiomyoma tissues.

Heritable genetic factors may contribute to the initiation and progression of endometriosis or leiomyoma (Treloar *et al.*, 1999). Gene polymorphisms are useful tools in the study of multifactorial disorders (Anderson *et al.*, 1994). Polymorphisms involved in steroid hormone biosynthesis and signalling may be useful genetic biomarkers for hormone-related diseases (Dunning *et al.*, 1999). Molecular geneticists are developing the third-generation human genome map with single-nucleotide polymorphisms (SNPs). Genetic studies of multifactorial

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disease such as endometriosis or leiomyoma are difficult because of the uncertainty of the polygenic trait. The identification of the related genes is essential for genetic diagnosis and gene therapy for genetic-associated disease. The analyses of SNPs can be implemented to analyse the mechanisms of complex genetic disorders.

ER is also involved in metabolic pathways influencing estrogen-related tissue growth and height stature (Schuit *et al.*, 2004a). ER α and ER β mediate much estrogen action. ER is a member of the nuclear receptor superfamily of ligand-activated transcription factors, which mediates estrogen actions in target tissues. Different polymorphisms have been described in ER α genes. Allelic variants of the gene encoding ER α and ER β may alter their expression and function, resulting in genetic variability. Several polymorphisms of ER α gene have been reported to be associated with alterations in receptor expression and function. The ER α gene, which is located on chromosome 6q25, contains some gene polymorphisms, including intron 1 polymorphisms *Xba*I (dbSNP: rs9340799) and *Pvu*II (dbSNP: rs2234693) (Ioannidis *et al.*, 2004; van Duijnhoven *et al.*, 2005). The associations between the ER α polymorphism and breast carcinoma or osteoporosis have been demonstrated (Liu *et al.*, 2001; Boyapati *et al.*, 2005).

Despite many epidemiological studies suggested that the ER α genetic variants confer increased susceptibility to individual disorders, few investigators demonstrated their association with endometriosis or leiomyoma. Reviewing the MEDLINE database, only two reports presented the non-association of ER α *Xba*I and *Pvu*II polymorphisms with the susceptibility of endometriosis (Wang *et al.*, 2004; Kim *et al.*, 2005). Furthermore, no investigators demonstrated correlations with leiomyoma. In our previous articles, we observed the correlation of endometriosis or leiomyoma with some hormone-related SNPs, including ER thymine-adenine (TA) dinucleotide repeat polymorphism (Hsieh *et al.*, 2003, 2005a), progesterone receptor Alu insertion (Hsieh *et al.*, 2005b) and androgen receptor trinucleotide polymorphism (Hsieh *et al.*, 2001, 2004). Herein, we tried to evaluate the distributions of ER α *Pvu*II and *Xba*I polymorphism in Taiwanese women with endometriosis or leiomyoma.

Materials and methods

Pre-menopausal Taiwanese women with surgically diagnosed severe endometriosis [Revised American Fertility Society (AFS) classification of endometriosis, 1985], leiomyoma and normal individuals without endometriosis and leiomyoma were recruited. All operations were performed by two surgeons (Y.-Y.H. and C.C.C.). Patients were divided into three groups: (i) severe endometriosis ($n = 112$); (ii) leiomyoma ($n = 106$) and (iii) normal controls ($n = 110$). The normal controls were recruited during annual health examination. The non-endometriosis or non-leiomyoma statuses were confirmed after detailed ultrasonography examination. The age of the patients in the three

groups was comparable (34.2 ± 3.8 versus 35.2 ± 4.1 versus 37.1 ± 4.8 years, respectively). This article was approved by the ethical committee and institutional review board of the China Medical University Hospital. Informed consents were signed by all women who donated their blood. All women accepted the peripheral blood sampling for genotype analyses.

The ER α gene polymorphisms were determined according to previously described methods (Kobayashi *et al.*, 1996; Lorentzon *et al.*, 1999). The ER α -351 *Xba*I A/G (uncuttable/cuttable) and -397 *Pvu*II T/C (uncuttable/cuttable) polymorphisms were assayed by the method of PCR and restriction fragment length polymorphism (RFLP). Genomic DNA was extracted from peripheral blood using Genomaker DNA extractor kit (Blossom, Taipei, Taiwan) and subjected to PCR, digestion with restriction enzymes and gel electrophoresis of the PCR products. Approximately 50 ng of genomic DNA was mixed with 20 pmol of PCR primer in a total volume of 25 μ l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each deoxyribonucleotide triphosphate and 1 unit of Amplitaq DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The PCR amplification was performed in a programmable thermal cycler GenAmp PCR system 2400 (Perkin Elmer Applied Biosystems). A 1374-bp fragment product, a part of intron 1 and exon 2 of ER α gene, was amplified by PCR (Kobayashi *et al.*, 1996; Lorentzon *et al.*, 1999). After PCR amplification, two ER α gene polymorphisms were analysed by restriction digestion with restriction enzymes (*Xba*I and *Pvu*II; New England Biolabs, Inc., Beverly, MA, USA). The primer sequences, PCR condition, base pairs for their wild and mutant types after RFLP are summarized in Table I. The SNP information for the genes involved was obtained through NCBI (<http://www.ncbi.nlm.nih.gov/LocusLink/>).

A 5- μ l PCR product was loaded into 1% agarose gel containing ethidium bromide for electrophoresis. Genotypes for *Xba*I and *Pvu*II polymorphisms were termed AA/AG/GG and TT/TC/CC, respectively. Genotypes and allelic frequencies for ER α *Xba*I A/G and *Pvu*II T/C gene polymorphisms in each group were compared. Correlations of ER α *Xba*I A/G and *Pvu*II T/C genotypes and endometriosis/leiomyoma were evaluated. Allelic frequencies are expressed as a percentage of the total number of alleles. The SAS system (version 8.1, SAS Institute Inc., Cary, NC, USA) with χ^2 test was utilized for statistical analyses. A P -value <0.05 was considered statistically significant.

Results

Genotype distribution and allele frequency of ER α -351 *Xba*I A/G and -397 *Pvu*II T/C gene polymorphisms between endometriosis/leiomyoma groups and normal controls were significantly different (Tables II–V). Genetic variations in the ER α (*Xba*I*G, *Pvu*II*C) were more prevalent in the disorder groups. Higher percentages of ER α mutant genotypes/alleles (*Xba*I*G, *Pvu*II*C) presented in the endometriosis/leiomyoma population were compared with normal controls. There was no statistically significant difference between the endometriosis and leiomyoma groups in the distributions of ER α *Xba*I and *Pvu*II polymorphisms. The most common genotypes and allele for ER α *Xba*I gene polymorphisms in each group were A-related genotypes and allele. ER α *Xba*I*G-related genotype and allele were associated

Table I. The primer sequences, PCR and restriction fragment length polymorphism (RFLP) conditions for estrogen receptor α (ER α)-351 A/G *Xba*I and -397 T/C *Pvu*II gene polymorphisms

Polymorphisms (locations)	Primers sequences (5'→3')	Denature (°C/s)	Annealing (°C/s)	Extension (°C/s)	Restriction enzyme (°C/min)	SNP sequence	Allelic variants	DNA fragment size (bp)
ER α -351 A/G <i>Xba</i> I polymorphism	F-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC	94/30	58/30	72/30	<i>Xba</i> I (37/60)	A (wild)	A	1374
ER α -397 T/C <i>Pvu</i> II polymorphism	R-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA	94/30	58/30	72/30	<i>Pvu</i> II (65/60)	G (mutant) T (wild)	G T	982 + 392 1374
						C (mutant)	C	937 + 437

SNP, single-nucleotide polymorphism.

Table II. Genotype distributions of estrogen receptor (ER)-351 A/G *XbaI* gene polymorphisms in women with endometriosis, leiomyoma and normal controls

Genotypes	Endometriosis (n = 112 ^{a,c})	Leiomyoma (n = 106 ^{a,b})	Normal controls (n = 110 ^{b,c})
AA homozygote	30 (26.8%)	21 (19.8%)	37 (33.6%)
AG heterozygote	64 (57.1%)	56 (52.8%)	71 (64.6%)
GG homozygote	18 (16.1%)	29 (27.4%)	2 (1.8%)

^aNot statistically different (endometriosis versus leiomyoma).

^bP < 0.00005 (leiomyoma versus controls).

^cP < 0.005 (endometriosis versus controls).

Table III. Allele frequencies of estrogen receptor (ER)-351 A/G *XbaI* gene polymorphisms in women with endometriosis, leiomyoma and normal controls

Allelic variants	Endometriosis (n = 224 ^{a,c})	Leiomyoma (n = 212 ^{a,b})	Normal controls (n = 220 ^{b,c})
A allele	124 (55.4%)	98 (46.2%)	145 (65.9%)
G allele	100 (44.6%)	114 (53.8%)	75 (34.1%)

A allele, wild uncuttable type; G allele, mutant cuttable type.

^aNot statistically different (endometriosis versus leiomyoma).

^bP < 0.00005 (leiomyoma versus controls).

^cP < 0.005 (endometriosis versus controls).

Table IV. Genotype distributions of estrogen receptor (ER)-397 T/C *PvuII* gene polymorphisms in women with endometriosis, leiomyoma and normal controls

Genotypes	Endometriosis (n = 112 ^{a,c})	Leiomyoma (n = 106 ^{a,b})	Normal controls (n = 110 ^{b,c})
TT homozygote	27 (24.1%)	25 (23.6%)	60 (54.5%)
TC heterozygote	68 (60.7%)	75 (70.8%)	44 (40.0%)
CC homozygote	17 (15.2%)	6 (5.6%)	6 (5.5%)

^aNot statistically different (endometriosis versus leiomyoma).

^bP < 0.00005 (leiomyoma versus controls).

^cP < 0.00005 (endometriosis versus controls).

Table V. Allele frequencies of estrogen receptor (ER)-397 T/C *PvuII* gene polymorphisms in women with endometriosis, leiomyoma and normal controls

Allelic variants	Endometriosis (n = 224 ^{a,c})	Leiomyoma (n = 212 ^{a,b})	Normal controls (n = 220 ^{b,c})
T allele	122 (54.5%)	125 (59.0%)	164 (74.5%)
C allele	102 (45.5%)	87 (41.0%)	56 (25.5%)

C allele, mutant cuttable type; T allele, wild uncuttable type.

^aNot statistically different (endometriosis versus leiomyoma).

^bP < 0.005 (leiomyoma versus controls).

^cP < 0.00005 (endometriosis versus controls).

with a higher susceptibility of endometriosis/leiomyoma. Proportions of ER α *XbaI**AA/AG/GG in each group were (i) 26.8/57.1/16.1%, (ii) 19.8/52.8/27.4% and (iii) 33.6/64.6/1.8%, respectively (Tables II and III). Proportions of ER α *XbaI**A/G alleles in each group were (i) 55.4/44.6%, (ii) 46.2/53.8% and (iii) 65.9/34.1%, respectively.

The most common genotypes and allele for *PvuII* gene polymorphisms in each group were T-related genotypes and allele. ER α *PvuII**C-related genotype and allele were associated with higher susceptibility of endometriosis/leiomyoma. Proportions of ER α *PvuII* TT/TC/CC in each groups were (i) 24.1/60.7/15.2%, (ii) 23.6/70.8/5.6% and (iii) 54.5/40.0/5.5%, respectively (Tables IV and V).

Proportions of ER α *PvuII* T/C alleles in each group were (i) 54.5/45.5%, (ii) 59.0/41.0% and (iii) 74.5/25.5%, respectively. These findings indicate that ER α *PvuII* and *XbaI* mutant genotype and allele are strongly associated with higher susceptibility of endometriosis or leiomyoma.

Discussion

Endometriosis and leiomyoma are both estrogen-dependent neoplasms of premenopausal women. Estrogen and ER play major roles in the pathogenesis of endometriosis and leiomyoma. Genetic defects and environmental factors including dietary and environmental regulating hormonal and non-hormonal conditions might contribute to the development of endometriosis and leiomyoma (Sano *et al.*, 1995). ER-related genotypes determine the function of the sex-steroid system not only at the receptor level but also at the level of hormone synthesis (Zofkova *et al.*, 2002).

The mechanisms of SNPs upon individual diseases remain uncertain. Unlike mutations, polymorphisms are not directly linked to certain diseases, but they are useful tools in the study of multifactorial disorders such as endometriosis and leiomyoma. Despite SNPs don't alter transcription, the disequilibrium of genotypes might influence the related 3D structure and efficiency of the transcripts (Shintani *et al.*, 1999; Kennon *et al.*, 2004; Shirasawa *et al.*, 2004). Intronic sequences have been reported to contain regulatory elements for transcription and splicing, giving rise to varying messenger RNA levels and different isoforms of mature messenger RNA, respectively (Gasch *et al.*, 1989; Carstens *et al.*, 1998). The ER polymorphisms might be in linkage disequilibrium with other unidentified functional gene variants, which cooperatively influence the susceptibility to endometriosis or leiomyoma.

Some polymorphic sites in the 5' region of the ER α gene have been demonstrated. ER α *14 TA or 12/13 TA repeats are associated with a higher risk of endometriosis or leiomyoma, respectively (Hsieh *et al.*, 2003, 2005a). The polymorphic sites defined by restriction enzymes (*PvuII* and *XbaI*) are located in the first intron of ER α gene (Kobayashi *et al.*, 1996). ER α *XbaI* and *PvuII* gene polymorphisms have been reported to be related to numerous estrogen-related diseases (Table VI). ER α -mutant alleles (*XbaI**G and *PvuII**C) were associated with elevated serum estradiol (E₂) production (Zofkova *et al.*, 2002; Schuit *et al.*, 2005). Such relationships provide the molecular pathways between ER α allelic variations and endometriosis/leiomyoma pathogenesis.

In this study, we observed that the genotype distributions and allele frequencies for ER α *XbaI* A/G and *PvuII* T/C polymorphisms were significantly different between the individuals with and without endometriosis/leiomyoma. Mutant variants for both ER α SNPs are correlated with higher susceptibility to endometriosis and leiomyoma. We hypothesize that both ER α *XbaI* and *PvuII* gene polymorphisms might predispose to endometriosis or leiomyoma development. The ER α *XbaI* -351*G-related genotype and allele are strongly related to the occurrence of leiomyoma, compared to being moderately correlated with the occurrence of endometriosis. In contrast, the ER α *PvuII* -397*C-related genetic variants are strongly correlated with endometriosis susceptibility, compared to being moderately correlated with leiomyoma risk.

In this study, we observed higher percentages of ER-351 *XbaI**G homozygote/allele and ER-397 *PvuII**T heterozygote and allele in the women with endometriosis or leiomyoma compared with normal controls. Mutant variants for both ER α SNPs were correlated with higher susceptibility of endometriosis or leiomyoma. Wild-type allele or homozygote might contribute to decreased illness risks. Our findings were compatible with some previous reports (Table VI), which suggested

Table VI. Correlations of estrogen receptor α (ER α)-351 *Xba*I and -397 *Pvu*II gene polymorphisms with individual diseases

SNP	Correlation	Non-correlation
	Diseases and references	Diseases and references
-351 <i>Xba</i> I and -397 <i>Pvu</i> II	Age of menarche (Stavrou <i>et al.</i> , 2006) Symptom of menopause (Malacara <i>et al.</i> , 2004) Breast cancer (Boyapati <i>et al.</i> , 2005; van Duijnhoven <i>et al.</i> , 2005) Osteoporosis (Lorentzon <i>et al.</i> , 1999; Massart, 2005) Height stature (Schuit <i>et al.</i> , 2004a) Osteoarthritis (Jin <i>et al.</i> , 2004) Prostate cancer (Hernandez <i>et al.</i> , 2006) Trigeminal neuralgia (Huang <i>et al.</i> , 2005) Adiposity (Huang <i>et al.</i> , 2005) Systemic lupus erythematosus (Johansson <i>et al.</i> , 2005) Cholesterol metabolism (Kajinami <i>et al.</i> , 2005) Alzheimer's disease (den Heijer <i>et al.</i> , 2004) Ischemic heart disease (Schuit <i>et al.</i> , 2004b) Paget's disease (Donath <i>et al.</i> , 2004) Aortic valve sclerosis (Nordstrom <i>et al.</i> , 2003)	Endometriosis (Wang <i>et al.</i> , 2004; Kim <i>et al.</i> , 2005) Menarche (Xu <i>et al.</i> , 2005) Osteoporosis (Dennison <i>et al.</i> , 2005; Jian <i>et al.</i> , 2005)
-351 <i>Xba</i> I	Clinical course of thalassemia (Iarussi <i>et al.</i> , 2005) Breast cancer (Lu <i>et al.</i> , 2005)	–
-397 <i>Pvu</i> II	Endometriosis (Georgiou <i>et al.</i> , 1999; Kitawaki <i>et al.</i> , 2001) Leiomyoma (Kitawaki <i>et al.</i> , 2001) Adenomyosis (Kitawaki <i>et al.</i> , 2001) Breast cancer (Onland-Moret <i>et al.</i> , 2005) Neurofibrillary tangles (Kazama <i>et al.</i> , 2004)	Breast cancer (Lu <i>et al.</i> , 2005)

SNP, single-nucleotide polymorphism.

that ER α *Xba*I and *Pvu*II genes might be associated with the clinical presentation of estrogen-related disorders. The correlations of ER α *Pvu*II T/C polymorphisms with endometriosis or leiomyoma were compatible with previous reports (Georgiou *et al.*, 1999; Kitawaki *et al.*, 2001).

We also observed that the distributions of ER α *Xba*I and *Pvu*II between endometriosis and leiomyoma were not significantly different, which was compatible with the report of Kitawaki *et al.* (2001). The similar distributions of ER α genotypes between these two estrogen-dependant disorders suggested their comparable underlying pathogenesis or molecular pathway. However, the distributions of ER α *Xba*I and *Pvu*II allelic variations in our study were not completely compatible with other studies (Kim *et al.*, 2005). These discrepancies might be due to different illness staging, severities, as well as racial or disease variations. In our study, we recruited individuals with severe endometriosis (AFS stage IV) instead of AFS stage I/II in Kim *et al.* (2005), which might have resulted in different distributions and conclusions.

There are controversial and inconsistent reports about the ER α -351 *Xba*I*G and -397 *Pvu*II*C-related genotype distributions or association in individual diseases among different races (Table VI). Ethnic variation plays a major role in genetic regulation of estrogen or ER activity and related polymorphism to individual diseases. Furthermore, the gender-specific influence of these gene polymorphisms should be considered in the relative studies. In the study of Lorentzon *et al.* (1999), the distributions of *Xba*I AA/AG/GG and *Pvu*II TT/TC/CC in 90 Caucasians boys were 8.9/40.0/51.1 and 22.2/44.5/33.3%, respectively. In contrast, in one study with 174 post-menopausal Korean women, Nam *et al.* (2005) demonstrated the related distributions were 3.5/29.3/67.2 and 14.9/46.0/39.1%, respectively. These incompetences might be due to racial, ethnic or gender variation as well as illness classification.

The mutant alleles for these two SNPs might be serving as markers of a functional variant in a nearby gene. This study could be extended to understand whether the ER α mutation also affects ER α function and endometriosis formation. After the clarification of their correlations

and role upon endometriosis and leiomyoma, ER α gene polymorphism may become a useful marker to predict the future susceptibility of these diseases and to permit early therapeutic intervention in women at high risk for endometriosis or leiomyoma. A further promising application of these polymorphisms comes from their pharmacogenomic implications, with the possibility of providing better guidance for therapeutic regimens, such as selective ER modulators and ER antagonist transfection therapy.

In conclusion, associations of ER α -351 *Xba*I and -397 *Pvu*II polymorphisms with endometriosis and leiomyoma exist. ER α *Xba*I*G- and *Pvu*II*C-related genotypes/alleles are correlated with higher susceptibilities of endometriosis or leiomyoma, which provided a useful tool in predicting endometriosis/leiomyoma susceptibility. ER α gene polymorphisms might influence the development of endometriosis or leiomyoma in Taiwanese women. They might be directly or indirectly correlated with the contributions to the pathogenesis of these gynaecological diseases. These findings provide a database for the further survey of the ER α polymorphisms in Asian individuals. Although the real role and mechanism of ER α gene polymorphisms upon these disorders have not yet been clarified, these polymorphisms deserve more attention to realize its importance to endometriosis/leiomyoma development.

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