

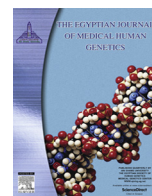
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Original article

Expression study of *CYP19A1* gene in a cohort of Iranian leiomyoma patientsLeila Emrahi¹, Javad Behroozi¹, Shirin Shahbazi^{1,*}

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ABSTRACT

Background: *CYP19A1* gene encodes aromatase, a microsomal key enzyme that catalyzes the synthesis of estrogens from androgens. Accumulating evidence has revealed that aromatase plays an important role in the pathogenesis of leiomyoma through increasing local concentration of estrogens. In this study, we examined the levels of *CYP19A1* mRNA to determine the impact of aromatase overexpression in uterine leiomyoma growth.

Subjects and methods: Tissues were obtained via myomectomy or hysterectomy from 30 patients. Total RNA was extracted and cDNA was synthesized from each frozen sample. Using SYBR Green dye, Real-time PCR assay was performed by sequence-specific primers. Relative mRNA expression was normalized to the mean of the Ct values determined for *HPRT1*. Gene expression ratio in each sample was determined relative to the mean Δ Ct value of tumor-free margin samples.

Results: PCR efficiencies for amplification reactions of *HPRT1*, and *CYP19A* genes were calculated as 0.93 and 0.96, respectively. Regression coefficients (R) for standard curves were above 0.90. The obtained data revealed that the mean fold increase of *CYP19A1* gene expression in leiomyoma samples relative to normal samples was 3.551 (95% CI: 0.04–6.64, S.E., 0.29–5.35).

Conclusions: Our results were in accordance with previous studies and imply that up-regulation of *CYP19A1* is correlated with the pathogenesis of leiomyoma tumors. We also observed that expression level of *CYP19A1* was not linked to the tumor size or localization. It can be concluded that; up-regulation of aromatase is a key factor in the initiation of tumor development as well as tumor growth.

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1. Introduction

Leiomyomas commonly known as fibroids are the most common benign tumors of uterus that are originated from smooth muscle cells [1]. Clinical symptoms of the disease may differ between patients from asymptomatic to severe complications which lead to hysterectomy [2]. Severity of the symptoms depends on the size and location of the lesions including: abdominal pain, prolonged bleeding, urinary incontinence and impairment of fertility [3].

The initial events in the development of fibroids are unknown, however, it is a well-known fact that estrogen and progesterone

play a central role in leiomyoma growth [4]. The fact that leiomyomas develop merely during the reproductive age confirms their dependence on ovarian steroids [5].

CYP19A1 also known as aromatase is a key enzyme involved in the estrogen biosynthesis. Aromatase is a member of the cytochrome P450 superfamily which is produced by various cells and tissues such as adipocytes, ovaries, placenta, endometriosis and uterine fibroids [6]. This enzyme is encoded by *CYP19A1* gene located at 15q21.2, contains nine coding exons. *CYP19A1* gene has several transcripts with alternative non-coding exon 1. The tissue-specific expression of aromatase is regulated by multiple promoters related to exons 1 which lead to alternative splicing in cells of gonadal and non-gonadal origin [7]. Overexpression of aromatase through promoter 1.4 has been shown in leiomyoma tissue [8]. Since *CYP19A1* mRNA strongly replicates the activity of aromatase enzyme it has been suggested that higher levels of aromatase in fibroids cause production of estrogen and tumor growth [9].

Pathogenesis of uterine fibroids is linked to several epidemiologic risk factors such as ethnicity [10]. Preceded by several publications, leiomyomas are three to nine times more prevalent

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Table 1

The sequence and characteristics of the primers used in the relative gene expression analysis.

Gene	Forward primer	Reverse primer	Amplicon size	Accession No.
<i>HPRT1</i>	CCTGGCGTCGTGATTAGTGATG	CAGAGGGCTACAATGTGATGGC	182 bp	NM_000194
<i>CYP19A1</i>	GGACACCTCTAACACGCTCTTC	GGTTTGATGAGGAGAGCTTG	105 bp	NM_001347256.1

in black women than in white women [11]. The disease typically develops at a younger age with more severe symptoms emphasizing the role of ethnicity and genetics [12].

In this study, we examined the expression levels of *CYP19A1* gene in leiomyoma samples of Iranian patients. The gene expression levels in tumors were compared with tumor-free margin samples to determine the impact of *CYP19A1* gene expression in uterine fibroids growths.

2. Subjects and methods

2.1. Subjects

The patients were examined by an expert gynecologist and evaluated according standard imaging procedures and laboratory analysis. Written informed consent approved by the ethics committee of the Tarbiat Modares University was obtained from each participant. Work has been carried out in accordance with the code of Declaration of Helsinki which is developed by the World Medical Association (WMA).

Tissue samples were obtained via myomectomy or hysterectomy from 30 patients. Each sample was sectioned into two replicates: one replicate was examined by a pathologist and the other replicate was transferred into liquid nitrogen containers for RNA extraction. The tissue samples were assigned as either leiomyoma tumors (n = 30) or tumor-free margin samples (n = 30) based on the pathological findings.

2.2. Quantitative real-time PCR analysis of *CYP19A1*

Total RNA was extracted from myoma samples using miR-Neasy[®] Mini kit (Qiagen, Germany) according to the kit instruction. High quality RNA samples (A260/280 > 1.8) were used as templates for cDNA synthesis using random hexamers (Protoscript[®] kit, New England BioLabs, USA). Using primer3 software exon-exon spanning primers were designed to specifically amplify *CYP19A1* cDNA. Primer sequences and amplicon sizes are indicated in Table 1. Real-time PCR assay was performed on StepOne Plus[®] instrument (Life sciences, USA) using SYBR Green dye. Relative mRNA expression in each tissue sample was normalized to the mean of the Ct values determined for Hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*). *HPRT1* as a common housekeeping gene for sample normalization is widely used in the qRT-PCR. Gene expression ratio in each sample was determined relative to the mean Δ Ct value of tumor-free margin samples.

2.3. Statistics

All data were analyzed using SPSS (version 20). The Kolmogorov-Smirnov test was used to evaluate the distribution of variables. T-test was used to compare differences between two independent that normally distributed and the Mann-Whitney U test was applied to not normally distributed data. p values < 0.05 were considered statistically significant.

Gene expression variations with more than twofold change were considered significant. The comparison between gene expression levels in tumor and tumor-free margin samples was

Table 2

Baseline demographic and clinical characteristics of the study subjects.

Variable	Mean \pm Std
Age (years)	40.93 \pm 4.8
BMI (kg/m ²)	25.1 \pm 3.2
Number of Pregnancies	2.1 \pm 0.9
Number of Deliveries	1.6 \pm 0.8
Number of Miscarriage	0.8 \pm 1.8
<i>Leiomyoma Localization</i>	<i>Proportion (%)</i>
Intramural	53.3%
Subserous	36.7%
Intramural & Subserous	10%
<i>Diameter of leiomyomas</i>	<i>Proportion (%)</i>
<2 cm	26.7%
2–5 cm	60.0%
>5 cm	13.3%
<i>Main complaint</i>	<i>Proportion (%)</i>
Pain	36.7%
Uterine abnormal bleeding	63.3%

performed using Relative Expression Software Tool (REST[®] 2009, Qiagen, Germany).

3. Results

3.1. Clinical characteristics of the patients

Clinical characteristics of the study subjects are indicated in Table 2. The patients ranged in age from 32 to 47 years, with a mean age of 40.93. The main complaint of the patients was uterine abnormal bleeding. The results yield no correlation with leiomyoma development and oral contraceptive usage, gravidity/parity, and body mass index.

3.2. Validation of relative real-time PCR assay

The specificity of Real-time PCR amplification process was verified by melting curve analysis of the amplified fragments. Melting curve analysis showed distinct single peaks for *HPRT1* (Tm = 82 °C) and *CYP19A1* (Tm = 80 °C) genes. There were no detectable amplification signals for non-template controls (Fig. 1). The accuracy of the quantitative assays was also verified by the generation of standard curves in LinReg (V.2014.5) software. PCR efficiencies for amplification reactions of *HPRT1*, and *CYP19A* genes were calculated as 0.93 and 0.96, respectively. Regression coefficients (R) for standard curves were above 0.90.

3.3. *CYP19A1* gene expression was up-regulated in clinical leiomyoma samples

The expression of *CYP19A1* gene was up-regulated in leiomyoma group (p = 0.047), which suggests the association of *CYP19A1* gene expression to the disease pathogenesis (Table 3 and Fig. 2). The mean fold increase of *CYP19A1* gene expression in leiomyoma samples relative to normal samples was 3.551 (95% CI: 0.04–6.64, S.E., 0.29–5.35). The comparison of each leiomyoma tissue to their matched normal myometrium showed that the expression of *CYP19A1* gene was up-regulated in 61% of the cases (Fig. 3).

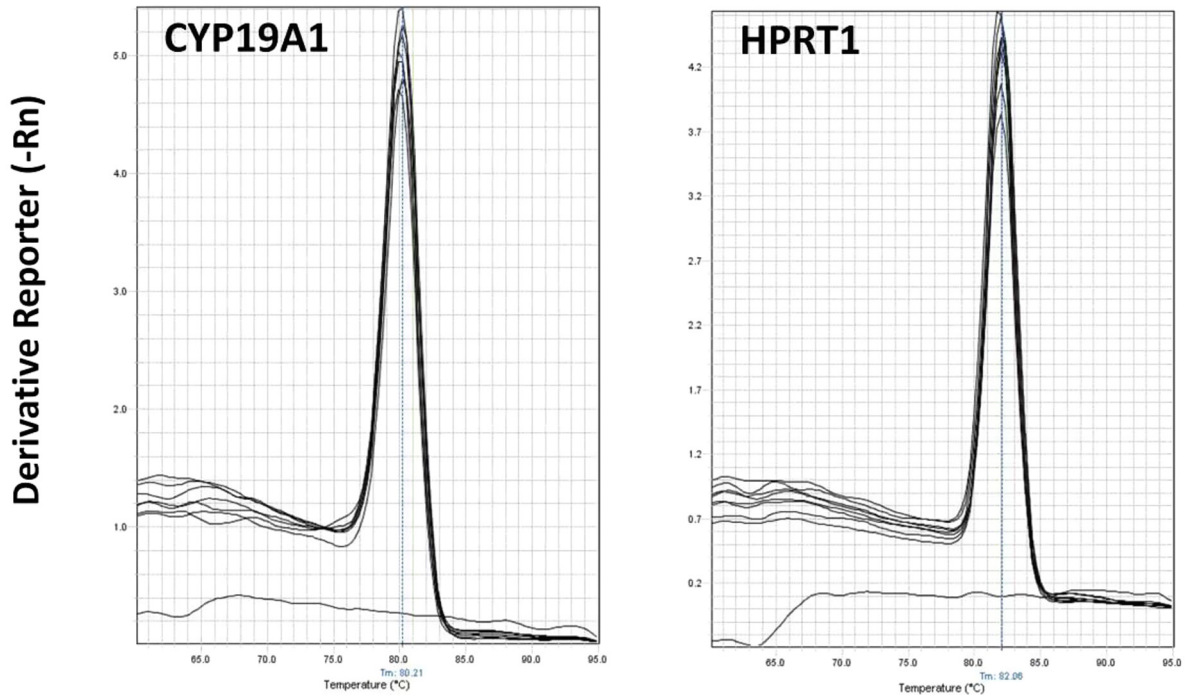


Fig. 1. The validation of Real-time PCR assays by melting curve analysis. Amplification of *HPRT1*, *CYP19A1* cDNA with corresponding gene specific primers was performed on StepOne Plus® Real-time PCR instrument. SYBR Green dye was included in the PCR reactions to detect the amplification of the desired fragments. Melting curve analysis showed a single specific peak for each gene which was indicated by their T_m values. No amplification curves were detected for NTC reactions after 40 PCR cycles.

Table 3

Results of the relative expression assay for *CYP19A1* gene in the leiomyoma cases. Ct values for *HPRT1* and *CYP19A1* genes were obtained by performing Real-time PCR on leiomyoma samples and their corresponding normal myometrium tissues. These data were transferred to REST analysis software and processed via $\Delta\Delta C_t$ method to determine the relative gene expression changes between the two groups.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	p value	Result
HPRT	REF	0.93	1.000				
CYP19A1	TRG	0.96	3.551	0.29–5.35	0.04–6.64	0.047	UP

CYP19A1 gene expression is up-regulated in leiomyoma sample group (in comparison to control group) by a mean factor of 3.551. REF; Reference gene, TRG; Target gene.

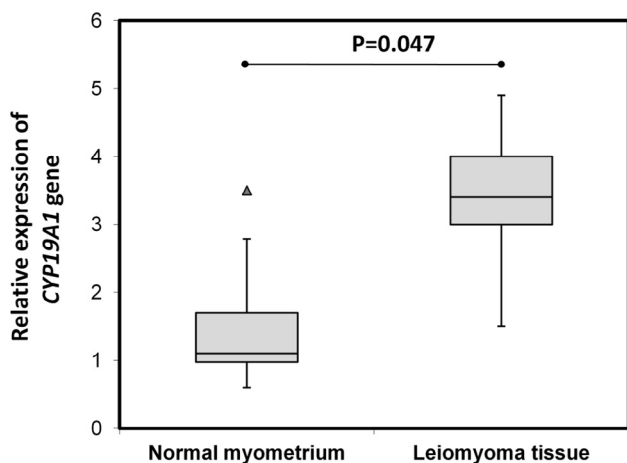


Fig. 2. The comparison of *CYP19A1* expression between leiomyoma and normal myometrium samples. Relative gene expression ratio of leiomyoma samples was compared to the corresponding values in normal myometrium group using $\Delta\Delta C_t$ method. Box-plots display the median and the distribution of the data. The expression level of the target gene was normalized to *HPRT1* mRNA in each group. ; Outlier data values.

Relative fold change of *CYP19A1* gene expression in leiomyoma samples

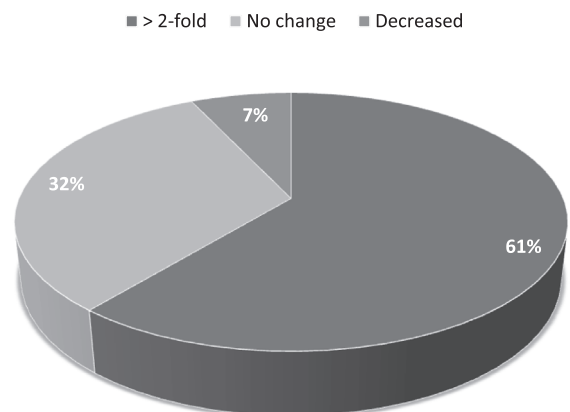


Fig. 3. The relative frequency of leiomyoma samples with deregulated *CYP19A1* transcript expression. The expression of *CYP19A1* gene in each leiomyoma sample was compared to its corresponding normal myometrium in the same patient. The distribution of *CYP19A1* transcript expression variations demonstrated the heterogeneity of the gene expression alterations among the leiomyoma patients.

Moreover, the variation of the gene expression in the studied patients had no significant correlation with the tumor size or location.

4. Discussion

Considerable evidence from clinical, epidemiological and experimental studies now supports the concept that development of uterine leiomyomas is dependent on the steroid hormones [11,13]. Accumulating data have revealed that aromatase play a pivotal role in the pathogenesis of leiomyoma through increasing local concentration of estrogens [14]. Early studies on leiomyoma tissues revealed an elevated enzyme level compared with normal myometrium [15]. Aromatase overexpression contributes to significant in situ conversion of androgen into estrogen active form which promotes the growth of tumor cells [16].

It has been shown that aromatase inhibitors reduce leiomyoma size in premenopausal and perimenopausal women [17]. A study by Sumitani et al. revealed that expression level of *CYP19A1* transcript and subsequent protein were up-regulated in leiomyoma tumors indicating that overexpression of *CYP19A1* is correlated to its protein product up-regulation [18].

Here we investigated the mRNA expression of *CYP19A1* in 30 pair myoma tumor and tumor-free margin tissues. Our results showed that *CYP19A1* transcript levels were 3.5-fold higher in leiomyomas compared to normal samples. This finding was in accordance with previous studies and imply that up-regulation of *CYP19A1* is correlated with the pathogenesis of leiomyoma [8,19]. We also observed that expression level of *CYP19A1* was not linked to the tumor size or localization. Based on these results it can be assumed that aromatase is responsible for the initiation of tumor growth and targeting inhibition of aromatase could be a potential prevention and therapy for leiomyoma.

Lots of efforts have been pushed towards the development of the aromatase inhibitor in uterine leiomyoma treatment [17]. It has been shown that aromatase inhibitor; letrozole significantly reduced tumor sizes and improved patient's symptoms without affecting bone mineral density [20]. Comparing aromatase inhibitor and gonadotropin-releasing hormone (GnRH) they recommended rapid onset of action with an aromatase inhibitor without initial gonadotropin for leiomyomas of any size [21]. Ishihara et al. assessed the impact of aromatase inhibitors on tumor size and symptoms of African-American women compared to other ethnic groups. As mentioned before, leiomyomas occur more frequently and at an earlier age in African-American women. They observed much higher *CYP19A1* mRNA levels in leiomyoma of African-American than other populations [19]. Since ethnicity is related to pathogenesis of leiomyoma, studies in different populations would be essential in order to develop more effective treatments.

5. Conclusion

Taking together, overexpression of aromatase is related to the pathogenesis of leiomyoma tumors, and its targeting inhibition could be a promising therapy for the prevention and treatment of this disease. Leiomyoma are responsible of increased invasive surgeries and development of such therapies significantly improves the women's quality of life.

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Disclosure

The authors have stated that they have no interests which might be perceived as posing a conflict or bias.

References

- [1] Vannuccini S, Clifton VL, Fraser IS, et al. Infertility and reproductive disorders: impact of hormonal and inflammatory mechanisms on pregnancy outcome. *Hum Reprod Update* 2016;22:104–15.
- [2] Wise LA, Laughlin-Tommaso SK. Epidemiology of uterine fibroids: from menarche to menopause. *Clin Obstet Gynecol* 2016;59:2–24.
- [3] Kashani BN, Centini G, Morelli SS, Weiss G, Petraglia F. Role of medical management for uterine leiomyomas. *Best Pract Res Clin Obstet Gynaecol* 2016;34:85–103.
- [4] Shahbazi S, Fatahi N, Amini-Moghaddam S. Somatic mutational analysis of MED12 exon 2 in uterine leiomyomas of Iranian women. *Am J Cancer Res* 2015;5:2441–6.
- [5] Zolfaghari N, Shahbazi S, Torfeh M, Khorasani M, Hashemi M, Mahdian R. Identification of differentially expressed k-ras transcript variants in patients with leiomyoma. *Reproduct Sci* 2017. 1933719116689596.
- [6] Bulun SE, Lin Z, Imir G, et al. Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. *Pharmacol Rev* 2005;57:359–83.
- [7] Harada N, Utsumi T, Takagi Y. Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. *Proc Natl Acad Sci USA* 1993;90:11312–6.
- [8] Shozu M, Sumitani H, Segawa T, et al. Overexpression of aromatase P450 in leiomyoma tissue is driven primarily through promoter I.4 of the aromatase P450 gene (*CYP19*). *J Clin Endocrinol Metab* 2002;87:2540–8.
- [9] Ishikawa H, Fencki V, Marsh EE, et al. CCAAT/enhancer binding protein beta regulates aromatase expression via multiple and novel cis-regulatory sequences in uterine leiomyoma. *J Clin Endocrinol Metab* 2008;93:981–91.
- [10] Blake RE. Leiomyomata uteri: hormonal and molecular determinants of growth. *J Natl Med Assoc* 2007;99:1170–84.
- [11] Commandeur AE, Styer AK, Teixeira JM. Epidemiological and genetic clues for molecular mechanisms involved in uterine leiomyoma development and growth. *Hum Reprod Update* 2015;21:593–615.
- [12] Othman EE, Al-Hendy A. Molecular genetics and racial disparities of uterine leiomyomas. *Best practice & research Clinical obstetrics & gynaecology*. 2008;22:589–601.
- [13] Donnez J, Donnez O, Dolmans MM. With the advent of selective progesterone receptor modulators, what is the place of myoma surgery in current practice? *Fertil Steril* 2014;102:640–8.
- [14] Marsh EE, Bulun SE. Steroid hormones and leiomyomas. *Obstet Gynecol Clin North Am* 2006;33:59–67.
- [15] Shozu M, Murakami K, Inoue M. Aromatase and leiomyoma of the uterus. *Semin Reprod Med* 2004;22:51–60.
- [16] Hilborn E, Stal O, Jansson A. Estrogen and androgen-converting enzymes 17beta-hydroxysteroid dehydrogenase and their involvement in cancer: with a special focus on 17beta-hydroxysteroid dehydrogenase type 1, 2, and breast cancer. *Oncotarget* 2017;8:30552–62.
- [17] Hilario SG, Bozzini N, Borsari R, Baracat EC. Action of aromatase inhibitor for treatment of uterine leiomyoma in perimenopausal patients. *Fertil Steril* 2009;91:240–3.
- [18] Sumitani H, Shozu M, Segawa T, et al. In situ estrogen synthesized by aromatase P450 in uterine leiomyoma cells promotes cell growth probably via an autocrine/intracrine mechanism. *Endocrinology* 2000;141:3852–61.
- [19] Ishikawa H, Reierstad S, Demura M, et al. High aromatase expression in uterine leiomyoma tissues of African-American women. *J Clin Endocrinol Metab* 2009;94:1752–6.
- [20] Gurates B, Parmaksiz C, Kilic G, Celik H, Kumru S, Simsek M. Treatment of symptomatic uterine leiomyoma with letrozole. *Reprod Biomed Online* 2008;17:569–74.
- [21] Parsanezhad ME, Azmoon M, Alborzi S, et al. A randomized, controlled clinical trial comparing the effects of aromatase inhibitor (letrozole) and gonadotropin-releasing hormone agonist (triptorelin) on uterine leiomyoma volume and hormonal status. *Fertil Steril* 2010;93:192–8.