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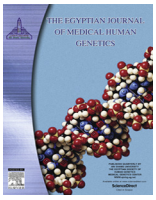

Original article

Expression study of *CYP19A1* gene in a cohort of Iranian leiomyoma patients

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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 ΔCt 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.

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 exons1 which lead to alternative splicing in cells of gonadal and non-gonadal origin [7]. Overexpression of aromatase through promoter I.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.
HPRT1	CCTGGCGTCGTGATTAGTGATG	CAGAGGGCTACAATGTGATGGC	182 bp	NM_000194
CYP19A1	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 *CYP19*A1 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 *CYP19*A1 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 miRNeasy® 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 ΔCt 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 performed using Relative Expression Software Tool (REST© 2009, Qiagen, Germany).

Table 2
Baseline demographic and clinical characteristics of the study subjects.

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

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 compliant 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).