AROMATASE EXPRESSION IN UTERINE LEIOMYOMATA IS REGULATED BY ALTERNATIVE PROMOTERS

AYSE GONCA IMIR, ZHIHONG LIN, SANTANU DEB, BERTAN YILMAZ, PING YIN, YOU-HONG CHENG, SERDAR BULUN,

Department of Obstetrics and Gynecology, Northwestern University Feinberg School of Medicine, 303 E Superior St, Chicago, IL

ABSTRACT

Objective: The objective is to determine the alternatively used promoters responsible for aromatase expression in uterine leiomyomata, which is the most common tumor in reproductive age.

Design: Uterine leiomyomata (fibroids), benign tumors of the uterus, are the leading cause of hysterectomy in the United States. Ovarian steroid hormones are essential for the progression of leiomyomata. Several studies demonstrated that aberrant expression of aromatase, the key enzyme responsible for estrogen biosynthesis, has been detected in leiomyoma tissue but not in disease-free myometrial tissue, thereby suggesting that leiomyoma cells synthesize estrogen *in situ*, which, in turn, contributes to the growth advantage of leiomyoma over surrounding myometrium. The CYP19 gene encoding aromatase (estrogen synthase) is expressed in several extragonadal sites and regulated in a tissue-specific fashion. Its regulatory region contains 10 tissue-specific promoters designated as I.1 (placenta-major), I.2 (placenta-minor), I.3 (adipose/breast cancer), I.4 (skin/adipose tissue), I.5 (fetal tissue), I.6 (bone), I.7 (vascular endothelial cell/breast cancer), PII (ovary/breast cancer/ endometriosis), 2.a (placenta-minor), and I.f (brain). Use of each promoter gives rise to incorporation of the promoter-specific first exon as the 5′-untranslated end of aromatase mRNA species. Thus, 5′-ends of aromatase mRNA species vary from tissue to tissue and may be viewed as the signature of the promoters used in this particular tissue. The aim of this study is to elucidate the promoters responsible for aromatase expression in leiomyoma tissue.

Materials and Methods: Human total RNA was isolated from leiomyoma (n=30), apparently normal myometrium 2 cm proximal to leiomyoma (n=8) samples and myometrium from disease-free uteri (n=5) samples from 28 patients.
5′-RACE: The unknown 5′-untranslated ends of aromatase mRNA were amplified. These sequences were cloned, sequenced and mapped to the genome.
REAL-TIME PCR: For validation of our 5′-RACE results, the expression of promoter-specific aromatase sequences were detected by real-time PCR in leiomyoma and myometrium tissues, in which various promoter-specific sequences were found by 5′-RACE.

Results: 5′-RACE:Aromatase mRNA sequences were cloned in 18 out of 30 (60 %) leiomyoma samples. The percentage of promoter-specific aromatase mRNA species in total number of positive samples was as follows: twelve promoter I.3 (67 %), four promoter II (23%), four promoter 2.a (23 %), four promoter I.6 (23 %), two promoter I.4-specific mRNA (11 %). One myometrial sample adjacent to leiomyoma contained promoter I.3-specific aromatase mRNA (12.5 %). No amplification was observed in the myometrium from disease-free uterus, as expected.
REAL-TIME PCR:Each promoter-specific aromatase mRNA (P II, I.3, I.7 or I.4) was illustrated as a percentage of total aromatase mRNA level that was independently determined.

Conclusion: Our present findings are strongly suggestive that the primary promoter region for aromatase expression in leiomyomata contains promoter II and Pr I.3. Promoters II and I.3 lie within 200 bp upstream of the common splice site in exon II and are coordinately regulated by a PGE2-cAMP-mediated signaling pathway in breast cancer, adipose tissue and endometriosis. Leiomyoma smooth muscle cell specific aromatase promoters giving rise to local estrogen formation and it may have significant impacts on human uterine leiomyomata physiology and pathology. Given the role of *in situ* estrogen in leiomyoma growth, inhibition of *in situ* expression of aromatase is a possible adjuvant therapy of conservative management, as in the case of breast cancer. Further investigation is required to identify transcriptional factors-mechanisms responsible for promoter-driven overexpression of aromatase P450 in uterine leiomyomata.

INTRODUCTION

Uterine leiomyomata (fibroids), benign tumors of the uterus, are the leading cause of hysterectomy in the United States (1). They are clinically significant in one third of women during reproductive age. They are 3-9 times more common in African-American women than in Whites (2). In considering the development of uterine leiomyomas, although the causes of the fibroids are unknown, epidemiological and experimental studies suggest that the development of leiomyomas are influenced by numerous factors including genetic, hormonal, and environmental influences (3). On the other hand, endocrinological studies have implicated that leiomyoma growth is up-regulated by circulating sex steroids, namely estrogen and progesterone. In addition to endocrine estrogen from the ovary, recent studies reported the possible contribution of in situ estrogen to leiomyoma growth in a paracrine, autocrine and intracrine manner (4). Leiomyomata express aromatase at higher levels than the surrounding myometrium and are able to synthesize estrogen. Estrogen synthesized in leiomyoma cells contributes to the growth advantage of leiomyoma over surrounding myometrium (5).
Aromatase, a member of the cytochrome P450 super-family, is a microsomal enzyme that catalyzes the conversion of androgens to estrogen (6). Human aromatase is encoded by CYP19 gene, a single-copy gene on 15q21.2. CYP19 comprises 10 exons, with exons II through X encoding the open reading frame of aromatase. Different first exons are known to encode unique 5′-untranslated regions of aromatase mRNA. Each first exon has its own upstream promoter region. First exons and corresponding promoters are used alternatively in a tissue- or cell-specific manner, enabling tissue- or cell-specific regulation of aromatase. The tissue-specific promoters located at the regulatory region designated as I.1 (placenta-major), I.2 (placenta-minor), I.3 (adipose/breast cancer), I.4 (skin/adipose tissue), I.5 (fetal tissue), I.6 (bone), I.7 (vascular endothelial cell/breast cancer), PII (ovary/breast cancer/ endometriosis), 2.a (placenta-minor), and I.f (brain) **(Fig 1).**
Use of each promoter gives rise to incorporation of the promoter-specific first exon as the 5′-untranslated end of aromatase mRNA species. Thus, 5′-ends of aromatase mRNA species vary from tissue to tissue and may be viewed as the signature of the promoters used in this particular tissue.
It has been reported in the previous study in Japanese population that the promoter I.4-specific aromatase mRNA species were dominantly present in most of the leiomyoma tissues obtained from six women (7).
Each mRNA species contains the identical coding region regardless of the variable untranslated first exon, however the encoded protein functions as the aromatase enzyme in each case. These give rise to transcripts of aromatase that differ only in the 5′-untranslated region. In this regard, identification of the promoter of aromatase P450 used in leiomyomas is the main step in understanding the mechanism of overexpression of aromatase P450 in leiomyoma tissues. The aim of the present study is to elucidate the promoters responsible for aromatase expression in leiomyoma tissue.

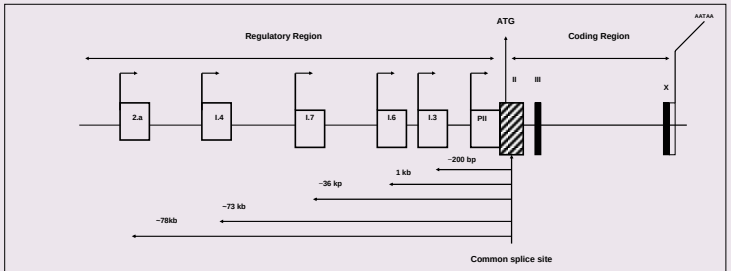

Fig 1. Schematic representation of location of each tissue-specific promoter of the CYP-19 gene

MATERIALS AND METHODS

Human total RNA was isolated from leiomyoma (n=30), apparently normal myometrium 2 cm proximal to leiomyoma (n=8) samples and myometrium from disease-free uteri (n=5) samples from 28 patients (age range, 31-42 yr) undergoing hysterectomy or myomectomy. These patients were African American (39%), Hispanic (25%) or White (22%).

5′-Rapid amplification of cDNA ends (5′-RACE)
The unknown 5′-untranslated ends of aromatase mRNA were amplified using the Smart RACE cDNA Amplification Kit (BD Biosciences Inc, USA). These sequences were cloned, sequenced and mapped to the genome

Real-time PCR
For validation of our 5′-RACE results, the expression of promoter-specific aromatase sequences were detected by real-time PCR in leiomyoma and myometrium tissues, in which various promoter-specific sequences were found by 5′-RACE. After reverse transcription of RNA, cDNA was amplified by adding Taqman master mix and optimized concentrations of primers and probes. Signals were monitored by an ABI Prism 7900 sequence detector. Sequence Detector Software SDS 2.1 (Applied Biosystems) was used for data analysis.

RESULTS

5′-Rapid amplification of cDNA ends (5′-RACE)
Aromatase mRNA sequences are cloned in 18 out of 30 (60 %) leiomyoma samples. The percentage of promoter-specific aromatase mRNA species in total number of positive samples was as follows: twelve promoter I.3 (67 %), four promoter II (23%), four promoter 2.a (23 %), four promoter I.6 (23 %), two promoter I.4-specific mRNA (11 %) **(Table I)**. One myometrial sample adjacent to leiomyoma contained promoter I.3-specific aromatase mRNA (12.5 %). No amplification was observed in the myometrium from disease-free uterus, as expected **(Fig 2).**

Real-time PCR
The usage of each promoter-specific aromatase mRNA (P II, I.3, I.7 or I.4) was illustrated as a percentage of total aromatase mRNA level that was independently determined (Figure 3).expression of aromatase P450 promoters such as Pr-II, Pr-I.3, Pr-I.4 and Pr-I.7 are demonstrated as the distribution of each promoter relative to the expression of aromatase P450 coding region **(Table II).**

Individually sequenced aromatase mRNA 5′-RACE clones from human uterine leiomyoma and myometrium tissues

Patient	Tissue	Race	Age	Mens cycle	Pr I.3	Pr II	Pr I.6	Pr 2.a	Pr I.4
1	LM LM	AA AA	39 39	Follicular Follicular	(+)	(+)		(+)	
2	LM MYO	White White	42 42	Follicular Follicular	(+) (+)				
3	LM	Hispanic	65	Postmen	(+)				
4	LM	AA	41	Luteal	(+)			(+)	
5	LM	White	42	Luteal					(+)
6	LM	Hispanic	42	Luteal	(+)				
7	LM	AA	40	Luteal		(+)	(+)		
8	LM	White	54	Postmen	(+)		(+)		
9	LM	AA	45	Follicular	(+)				
10	LM	Hispanic	54	Postmen			(+)	(+)	
11	LM	White	38	Follicular					(+)
12	LM	AA	37	Follicular	(+)				
13	LM	AA	39	Follicular	(+)	(+)	(+)		
14	LM	AA	36	Luteal	(+)				
15	LM	AA	45	Follicular	(+)	(+)			
16	LM	AA	42	Luteal				(+)	
17	LM	White	42	Follicular	(+)				

Table I. Aromatase mRNA sequences cloned by 5′-RACE in leiomyoma and myometrium tissues (AA, African American)

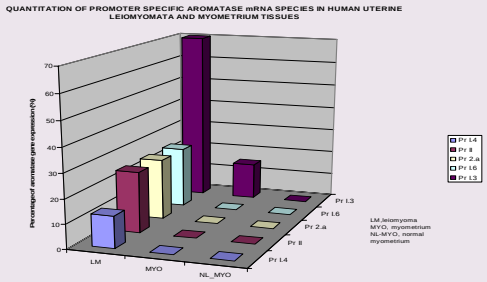

Fig 2. Percentage of aromatase transcripts with specific 5′-untranslated ends (exon Is) in 5′-RACE.

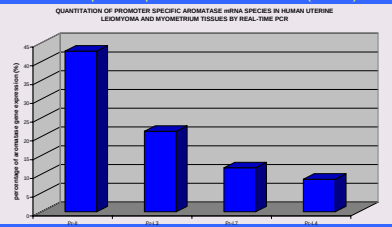

Fig 3. Real-time PCR quantification of aromatase gene expression in leiomyoma and myometrium samples in which various promoter-specific sequences are found during 5′-RACE.

DISCUSSION

Our present findings are strongly suggestive that the primary promoter region for aromatase expression in leiomyomata contains promoter II and Pr I.3. We found slightly different results in 5′-RACE and real-time PCR experiments. For example, real-time PCR showed higher usage of promoter II. This may be due to a more skewed representation of promoter usage as a result of 5′-RACE procedure. One potential source may be varying transformation and ligation efficiency in different promoters of aromatase during 5′-RACE. Promoters II and I.3 lie within 200 bp upstream of the common splice site in exon II and are coordinately regulated by a PGE2-cAMP-mediated signaling pathway in breast cancer, adipose tissue and endometriosis. In contrast to recently published reports on tissues from Japanese patients, we found a very limited use of promoter I.4, which is different than promoter I.3 in terms of regulation, tissue-specificity and sequence. The discrepancy of the results in two studies may be due racial differences.

CONCLUSIONS

Leiomyoma smooth muscle cell specific aromatase promoters giving rise to local estrogen formation with potential intracrine and paracrine effects may have significant impacts on human uterine leiomyomata physiology and pathology. Given the role of *in situ* estrogen in leiomyoma growth, inhibition of *in situ* expression of aromatase is a possible adjuvant therapy of conservative management, as in the case of breast cancer. Further investigation is required to identify transcriptional factors-mechanisms responsible for promoter-driven overexpression of aromatase P450 in uterine leiomyomata.

Supported by NIH award HD46260

REFERENCES

1- Sreekantaiah C, Ladanyi M, Rodriguez E, Chaganti RS 1994 Chromosomal aberrations in soft tissue tumors. Relevance to diagnosis, classification, and molecular mechanism. Am J Pathol 144: 1121-1134.
2- Mattingly RF, Thompson JD. 1985. Leiomyomata uteri and abdominal hysterectomy. In: Mattingly RF, Thompson JD, eds. Operative gynecology, Philadelphia: Lippincott; 203-255.
3- Ross RK, Pike MC, Vessey MP, Bull D, Yeates D, Casagrande JT 1986 Risk factors for uterine fibroids: reduced risk associated with oral contraceptives. Br Med J 293: 359-362.
4- Bulun SE, Simpson ER, Word RA 1994 Expression of the CYP19 gene and its product aromatase cytochrome P450 in human uterine leiomyoma tissues and cells in culture. J Clin Endocrinol Metab 78: 736-743.
5- Sumitani H, Shozu M, Segawa T, Murakami K, Yang H-J, Shimada K, Inoue M 2000 In situ estrogen synthesized by aromatase P450 in uterine leiomyoma cells promotes cell groth probably via an autocrine/paracrine mechanism. Endocrinology 141:3852-3861.
6- Nebert DW, Nelson DR, Adesnik M et al. 1989 The P450 gene superfamily : recommended nomenclature. DNA. 8:1-13.
7- Shozu M, Sumitani H, Segawa T, et al. 2001 Overexpression of aromatase P450 in leiomyoma tissues is driven through the promoter I.4 of aromatase P450. J Clin Endocrinol Metab; 86: 5405-5411.
8- Sebastian S, Takayama K, Shozu M, Bulun SE 2002 Cloning and characterization of a novel endothelial promoter of the human CYP19 (aromatase P450) gene that is up-regulated in breast cancer tissue. Mol Endocrinol. Oct; 16(10):2243-54.