

## Down-regulation and gene hypermethylation of the 14-3-3 gamma in uterine leiomyoma

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### 1. ABSTRACT

We demonstrated that the expression of 14-3-3 gamma was lower in uterine leiomyomas compared to the adjacent normal myometrium. Here, we show that 14-3-3 gamma promoter is methylated more in leiomyomas than myometrium. The level of 14-3-3 gamma protein in leiomyomas did not change in respect to age, size, location, or the type of leiomyoma. ER-alpha, ER-beta, and PR proteins were also higher in leiomyomas and the level of these proteins negatively correlated with the level of 14-3-3 gamma protein. These results suggest that the hypermethylation of the 14-3-3 gene promoter accounts for the decreased 14-3-3 gamma in leiomyomas and that such a low level of expression may be involved in the pathogenesis of leiomyomas.

### 2. INTRODUCTION

The 14-3-3 protein is a member of a highly conserved acidic protein family implicated in the regulation of a variety of cellular processes, such as cell cycle progression, apoptosis, signal transduction, and malignant transformation (1). This protein family is also involved in the development of different types of cancer. In humans, seven genes encode different 14-3-3 isoforms, beta, gamma, epsilon, sigma, zeta, theta, and eta (2). To date, the involvement of 14-3-3 proteins in cancer has been most thoroughly investigated for the sigma and zeta isoforms. The expression of 14-3-3 sigma protein has been investigated in glioma (3), endometrial carcinoma (4), breast cancer (5), ovarian cancer (6), and lung cancer (7). Wang *et al.* (8) examined 14-3-3

sigma protein levels in uterine leiomyoma tissues using immunohistochemistry, and observed their decrease, compared with normal myometrium. Moreover, loss of 14-3-3 sigma gene expression was related to the hypermethylation of CpG islands in breast and lung cancers (5, 7). On the other hand, the 14-3-3 zeta protein is expressed in breast (9), stomach (10), and lung cancers (11, 12), and oral squamous cell carcinoma (13).

Studies of the 14-3-3 gamma protein have usually focused on breast cancer and glioma (3, 14), while little is known about its role in gynecological cancers. In our previous study (15), using proteomic techniques, we observed differences in abundance of 14-3-3 gamma protein between uterine leiomyoma and normal myometrium. However, the molecular mechanisms underlying the regulation of 14-3-3 gamma gene expression and the clinical implications of this protein in uterine leiomyomas, e.g., relationship with the menstrual cycle, clinicopathological characteristics, and progesterone receptor (PR) and estrogen receptor (ER) status, remain unclear.

Benign uterine leiomyomas impose a great health burden in reproductive-age women because of the associated high morbidity. Uterine leiomyoma increases the risk of abnormal bleeding, infertility, and pregnancy complications (16). It is generally accepted that this is a hormone-dependent disease, and that estrogen and progesterone greatly influence its development (17-19) through the activation of ER and PR, respectively. Previous studies indicated that ER-alpha and ER-beta might play different roles in the proliferation of uterine leiomyoma cells. One study (20) reported that the expression levels of ER-alpha, ER-beta, and PR genes are higher in premenopausal uterine leiomyomas than in the adjacent normal myometrium. However, another study (21) found no difference in ER-alpha and ER-beta gene transcript levels in these tissues. Furthermore, this study reported higher levels of expression of ER-alpha gene than ER-beta gene in leiomyoma.

In the present study, we explored the epigenetics of 14-3-3 gamma gene expression in uterine leiomyomas, focusing on promoter methylation. We investigated 14-3-3 gamma protein levels in uterine leiomyoma and in the adjacent normal myometrium during menstrual cycle. Further, we explored the relationships between 14-3-3 gamma protein levels and the clinicopathological characteristics of uterine leiomyomas, and the relationship between 14-3-3 gamma and ER-alpha, ER-beta, and PR proteins.

## 3. MATERIALS AND METHODS

### 3.1. Patients

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University and conforms to the declaration of

Helsinki. Written informed consent was obtained from each subject.

Uterine leiomyoma and adjacent normal myometrial tissues were obtained from 65 women 35 to 56 years old) who underwent hysterectomy for symptomatic leiomyomas. Normal myometrial tissue specimens were obtained at surgery and were sampled at sites distant from the tumor site. Tissues were stored at -80 °C until use. Pathological diagnosis and phase of the menstrual cycle were established by the Department of Pathology. Leiomyoma was the only histopathological finding in the uterine tissues in all the subjects. The following leiomyoma characteristics were included in the analyses: size (based on the longest diameter, d, in cm,  $d < 4$ ,  $4 \leq d < 6$ ,  $6 \leq d < 8$ ,  $8 \leq d < 10$ , or  $d \geq 10$ ), type (i.e. multiple or single), and location (submucosal, intramural, or subserosal, based on ultrasound examination).

All subjects were free of infections, adenomyosis, metabolic disorders, and other malignancies. None of the subjects had received hormones, hormone analogs, or gonadotropin-releasing hormone analogs three months prior to surgery.

### 3.2. Sodium bisulfite conversion and methylation-specific polymerase chain reaction (MSP)

Genomic DNA was isolated using Ezup Genomic Animal DNA Isolation Kit (Sheneng Bicolor, Shanghai, China) according to the manufacturer's instructions. Unmethylated cytosines were converted to uracils in DNA samples (2 mg) using EpiTect Bisulfite Kit (Qiagen, Frankfurt, Germany), following recommended procedures. MSP primers were designed with MethPrimer (<http://www.urogene.org/methprimer/>). Primers were as follows: 14-3-3 non-methylation-specific primers: 5'-TGAGTAATTGGTGTAGAAAGTTTGG-3' and 5'-CCCTCAATAAACAAAACAAAACAAT-3'; methylation-specific primers: 5'-GCGAGTAATTGGTGTAGAAAGTTC-3' and 5'-CTCAATAAACGAAACGAAACGAT-3'. MSP mixtures contained 4 µL bisulfite-treated DNA (2 mg), 20 pmol of each primer, 17 µL RNase-free water, and 25 µL EpiTect Master Mix, in 50 µL final reaction volume. All MSP reactions were performed under the following conditions: 95 °C for 10 min; followed by 40 cycles of: 94 °C for 30 s, 54 °C (methylation-non-specific primers) or 53 °C (methylation-specific primers) for 45 s, 72 °C for 45 s; and final extension at 72 °C for 10 min. MSP products were analyzed by electrophoresis in 2% agarose gels.

### 3.3. Bisulfite sequencing analysis

To confirm the accuracy of the MSP assay, bisulfite sequencing of purified MSP-PCR products was performed at Invitrogen core facility (Carlsbad, CA, USA). The sequencing revealed full cytosine conversion to uracil in non-CpG dinucleotide contexts, confirming the specificity of the bisulfite reaction.

**Table 1.** Differences in the expression of 14-3-3 gamma in leiomyoma and myometrium during the menstrual cycle [median (P25–P75)]

	N	Age	Leiomyoma	Myometrium	P <sup>a</sup>
Proliferative phase	37	48 (46–50)	1.50 (1.15–1.95)	1.95 (1.44–2.25)	0.001
Secretory phase	22	46 (42–51)	1.64 (1.25–1.93)	1.93 (1.79–2.66)	0.008
Atrophic phase	6	55 (53–56)	2.41 (1.13–3.37)	1.71 (1.36–3.35)	1.0
Total	65	48 (35–56)	1.60 (1.17–1.97)	1.94 (1.58–2.38)	0.000
P <sup>b</sup>			0.290	0.777	
P <sup>a</sup> differences between uterine leiomyoma and adjacent normal myometrium, P <sup>b</sup> differences among different phases in uterine leiomyoma or in myometrium					

### 3.4. Western blot analysis

Tissue samples (50–100 mg) were homogenized in Radioimmunoprecipitation Assay Lysis buffer (Protein Assay Reagent Kit, Biyuntian, Shanghai, China) and cleared by centrifugation at 13,000 g at 4 °C for 20 min. Protein concentrations were determined by the bicinchoninic acid method (Protein Assay Reagent Kit, Biyuntian). Protein samples (30 µg) were separated on sodium dodecyl sulfate polyacrylamide gels (12% for 14-3-3 gamma protein, 10% for ER-alpha and ER-beta proteins, and 8% for PR proteins), and electrophoretically transferred with wet transfer onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline containing 20% Tween 20 and 10% skim milk, membranes were incubated with either monoclonal mouse anti-14-3-3 gamma protein antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-ER-alpha antibody (1:800 dilution, Bioworld, Minneapolis, MN, USA), polyclonal rabbit anti-ER-beta antibody (1:800, Santa Cruz Biotechnology), or polyclonal rabbit anti-PR antibody (1:800 dilution, Bioworld), at 4°C overnight. After extensive washing, horseradish peroxidase-conjugated anti-IgG secondary antibodies (goat anti-mouse IgG, 1:1000 dilution, for 14-3-3 gamma protein detection, Biyuntian; goat anti-rabbit IgG, 1:1000 dilution, Bioworld, for the remaining proteins) were added, and incubated for 1 h at room temperature. The reactions were developed using enhanced chemiluminescence assay (BeyoECL Plus, Axygen, USA). To obtain protein loading controls for 14-3-3 gamma protein, the blots were stripped and tubulin was detected with a polyclonal mouse anti-tubulin antibody (1:1000 dilution, Biyuntian) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:1000 dilution, Biyuntian). Because of the different protein antibody molecular sizes, for protein loading controls for ER alpha, ER beta and PR, each blot was re-probed using a polyclonal mouse anti-GAPDH primary antibody (1:1200 dilution, Biyuntian) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:1000 dilution, Biyuntian). Each experiment was repeated at least three times.

### 3.5. Immunohistochemistry

Tissue sections (4 µm) were cut from paraffin blocks, deparaffinized in xylene, and rehydrated through a graded alcohol series. The slides were microwaved in citrate buffer for 5 min to facilitate antigen retrieval. Sections were incubated for 20 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and blocked with normal goat serum (10%). This was followed by incubation with primary monoclonal mouse antibody raised against human 14-3-3 gamma protein (1:100 dilution, Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature, followed by incubation with biotinylated goat anti-mouse antibody for 20 min (1:10 dilution, Biyuntian, China). Streptavidin peroxidase (3%, GIBCO, Grand Island, NY, USA) and 3'3'-diaminobenzidine tetrahydrochloride (DAB) (1:50 dilution, GIBCO) were used for signal detection. Sections were counterstained with hematoxylin.

### 3.6. Statistical analyses

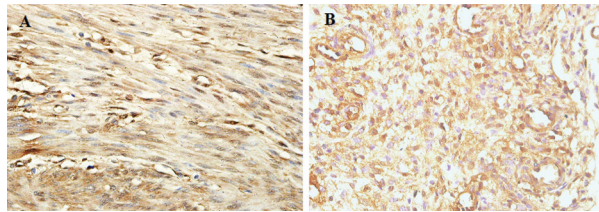
All statistical analyses were done using SPSS version 17.0 (IBM, Chicago, IL, USA). Data are expressed as medians (25<sup>th</sup>–75<sup>th</sup> percentiles, P25–P75). Differences of patient characteristics at baseline in different tumor stages were assessed using Kruskal–Wallis H test. Methylation frequencies of 14-3-3 gamma gene promoter in uterine leiomyoma and normal myometrium were compared using  $\chi^2$  test. Differences in leiomyomal and myometrial protein levels were analyzed by Wilcoxon test. Spearman's rank method was used to calculate correlation coefficients. P<0.05 was considered statistically significant.

## 4. RESULTS

### 4.1. Patient characteristics

Patients' characteristics are given in Table 1. From the 65 subjects (median age 48 years), 37 were in the proliferative phase of the menstrual cycle (median age 48 years), 22 in the secretory phase (median age 46 years), and six in the atrophic phase (median age 55 years).

### 14-3-3 gamma in uterine leiomyomas



**Figure 1.** The methylation status of 14-3-3 gamma in leiomyoma and myometrium. A: The MSP result for 14-3-3 gamma in leiomyoma and myometrium. B: Sequencing results of cloned MSP products from leiomyoma and myometrium. M: myometrium, Ly: leiomyoma, mdna: control DNA only with methylated DNA, udna: control DNA only with unmethylated DNA, blank circle: unmethylated, black circle: methylation, blank location: no expression.

**Table 2.** Comparison of the methylation frequency of 14-3-3 gamma in leiomyoma and in myometrium during the menstrual cycle (%)

	Methylation status		
	Complete methylation	Partial methylation	Unmethylated
Total			
Myometrium	20.0 (13/65)	23.1 (15/65)	56.9 (37/65)
Leiomyoma	30.8 (20/65)	35.4 (23/65)	33.8 (22/65)
$P=0.030$			
Proliferative phase			
Myometrium	16.2 (6/37)	18.9 (7/37)	64.9 (24/37)
Leiomyoma	27.0 (10/37)	29.7 (11/37)	43.2 (16/37)
$P=0.175$			
Secretory phase			
Myometrium	22.7 (5/22)	27.3 (6/22)	50.0 (11/22)
Leiomyoma	40.9 (9/22)	31.8 (7/22)	27.3 (6/22)
$P=0.260$			
Atrophic phase			
Myometrium	33.3 (2/6)	33.3 (2/6)	33.3 (2/6)
Leiomyoma	16.7 (1/6)	16.7 (1/6)	66.7 (4/6)
$P=0.513$			
Proliferative+secretory			
Myometrium	18.6 (11/59)	22.0 (13/59)	59.3 (35/59)
Leiomyoma	32.2 (19/59)	32.2 (19/59)	35.6 (21/59)
$P=0.034$			

## 4.2. Tissue methylation status of 14-3-3 gamma gene promoter and protein tissue levels

### 4.2.1. 14-3-3 gamma gene promoter methylation status

MSP assays were performed to assess the methylation of 14-3-3 gamma gene promoter in

uterine leiomyoma and normal myometrium. Figure 1 shows MSP results, confirmed by bisulfite sequencing analysis. In uterine myeloma, 30.8% 14-3-3 promoters were completely methylated, 35.4% were partially methylated, and 33.8% were unmethylated (Table 2). In comparison, in normal myometrium, 20.0% promoters were completely methylated, 23.1% partially methylated, and 56.9% completely unmethylated (Table 2). Thus, methylation frequency of the 14-3-3 gamma gene promoter in uterine leiomyoma was significantly higher than in normal myometrium ( $P=0.030$ ) (Figure 2). When women in the proliferative and secretory menstrual phases were considered together, similar tendency was found ( $P=0.034$ ) (Figure 2).

### 4.2.2. Cellular distribution of 14-3-3 gamma protein and tissue levels

Immunohistochemistry revealed that cellular distribution of 14-3-3 gamma protein did not significantly differ between uterine leiomyoma and myometrium. 14-3-3 gamma protein was detected in the cytoplasm of both leiomyoma and myometrial cells (Figure 3). Nevertheless, Western blot analysis indicated significantly decreased 14-3-3 gamma protein levels in uterine leiomyoma compared to adjacent normal myometrium tissue ( $P<0.05$ ) (Figures 4 and 5). When specific phases of the menstrual cycle were considered, 14-3-3 gamma protein levels were lower in uterine leiomyoma than in normal myometrium in the proliferative and secretory menstrual phases only (Table 1, Figure 5).

### 4.2.3. Relationship between 14-3-3 gamma gene promoter methylation and 14-3-3 protein levels

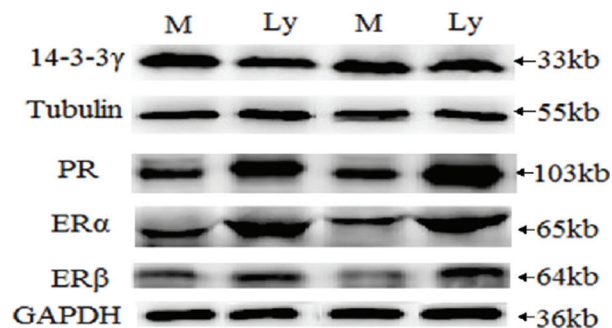
No significant correlation was found between the methylation status of 14-3-3 gamma gene promoter and 14-3-3 protein levels in the myometrium ( $r_s=-0.074$ ,  $P=0.636$ ). In contrast, the methylation frequency of 14-3-3 gamma gene promoter negatively correlated with 14-3-3 gamma protein levels in leiomyoma tissue, particularly in the proliferative ( $r_s=-0.350$ ,  $P=0.045$ ) and secretory ( $r_s=-0.590$ ,  $P=0.034$ ) menstrual phases. When women in the proliferative and secretory menstrual phases were considered together, similar tendency was found ( $r_s=-0.470$ ,  $P=0.038$ ).

## 4.3. Relationships between 14-3-3 gamma protein tissue levels and individual clinicopathological characteristics

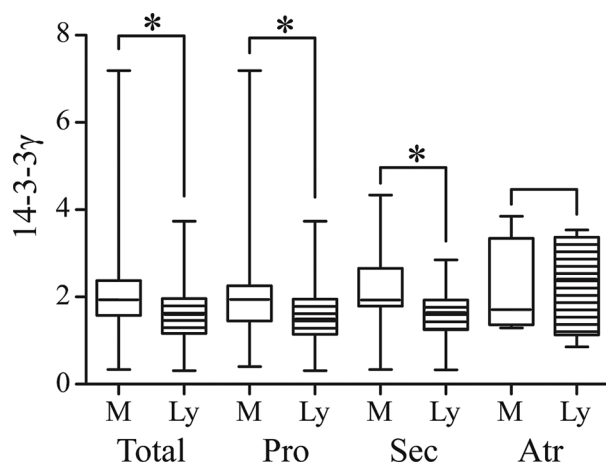
### 4.3.1. 14-3-3 gamma protein levels vs. patient age

When women in age groups <48 years old and  $\geq 48$  years old were compared, no significant differences in leiomyoma 14-3-3 gamma protein levels were detected. Likewise, myometrial 14-3-3 gamma protein levels were similar between the age groups. We compared leiomyomal with myometrial levels of 14-3-3

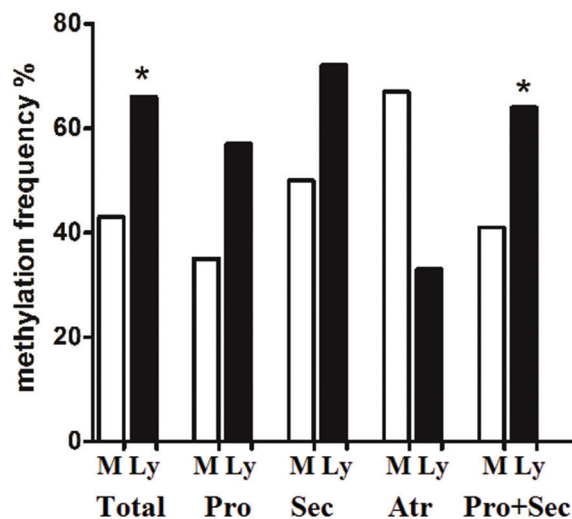




**Figure 2.** The methylation frequency of 14-3-3 gamma in leiomyoma and myometrium during the menstrual cycle. M: myometrium, Ly: leiomyoma, pro: proliferative phase, sec: secretory phase, atr: atrophic phase, \* $P < 0.05$ .



**Figure 3.** The distribution of 14-3-3 gamma in leiomyoma and myometrium (SP staining,  $\times 400$ , proliferative phase). A: myometrial tissue; B: leiomyoma tissue.



**Figure 4.** The protein expression of 14-3-3 gamma, PR, ER alpha, and ER beta in leiomyoma and myometrium (proliferative phase). M: myometrium, Ly: leiomyoma.

gamma proteins between different age groups. In women younger than 48 years old, leiomyoma 14-3-3 gamma protein levels were significantly lower than myometrial levels, whereas no significant difference was found in women older than or at 48 years old (Table 3).

#### 4.3.2. 14-3-3 gamma protein levels vs. uterine leiomyoma size

14-3-3 gamma protein levels in uterine leiomyomas of different sizes were comparable. Similar results were found in the corresponding myometrium samples. In patients where leiomyoma sizes were  $4 \leq d < 6$  cm or  $6 \leq d < 8$  cm, leiomyoma 14-3-3 gamma protein levels were significantly lower than myometrial 14-3-3 gamma protein levels (Table 3).

#### 4.3.3. 14-3-3 gamma protein levels vs. leiomyoma type

Leiomyoma levels of 14-3-3 gamma protein were comparable in women with multiple or single leiomyomas. Similar results were found in these patient groups for myometrial 14-3-3 gamma protein levels. In women with multiple leiomyomas, leiomyoma levels of 14-3-3 gamma proteins were lower than myometrial 14-3-3 gamma protein levels (Table 3).

#### 4.3.4. 14-3-3 gamma protein levels vs. leiomyoma location

Patients with subserosal, submucosal, or intramural leiomyomas, had comparable levels of leiomyoma 14-3-3 gamma protein. This was similar to what we observed with 14-3-3 gamma protein myometrial levels in these patients. Compared with patients with normal myometrium, patients with intramural leiomyomas were characterized by lower leiomyoma and myometrial 14-3-3 gamma protein levels (Table 3).

#### 4.4. Levels of ER and PR proteins in uterine leiomyomas

##### 4.4.1. ER-alpha, ER-beta and PR protein tissue abundances during the menstrual cycle

Among all patients, levels of ER-alpha, ER-beta, and PR proteins were generally higher in leiomyoma compared to the myometrium (Table 4, Figure 4). In both, proliferative and secretory phases of menstrual cycle, PR and ER-beta protein levels in uterine leiomyomas were significantly higher than in myometrium. No such significant difference was found in levels of these proteins during the atrophic phase. In contrast, ER-alpha protein levels were higher in leiomyoma than in myometrial tissue only in the proliferative phase.

##### 4.4.2. 14-3-3 gamma expression vs. ER-alpha, ER-beta, and PR protein levels in uterine leiomyoma patients

14-3-3 gamma protein levels negatively correlated with ER-alpha protein levels in uterine

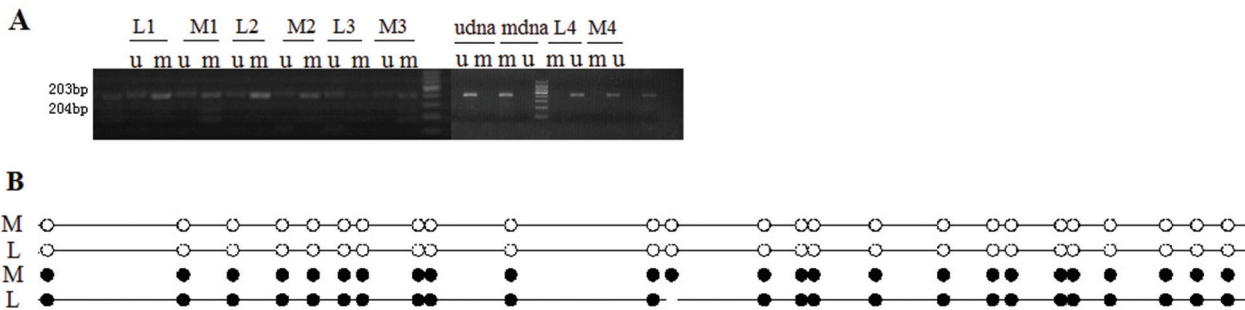


Figure 5. The expression of 14-3-3 gamma in leiomyoma and myometrium during the menstrual cycle. M: myometrium, Ly: leiomyoma, pro: proliferative phase, sec: secretory phase, atr: atrophic phase, \*P<0.05.

**Table 3.** The correlation between 14-3-3 gamma expression in leiomyoma and myometrium and clinicopathological characteristics [median (P25–P75)]

	N	Leiomyoma	Myometrium	P <sup>a</sup>
Age				
≥48	35	1.60 (1.29–1.96)	2.00 (1.80–2.21)	0.053
<48	30	1.55 (0.88–1.90)	1.92 (1.11–2.79)	0.003
P <sup>b</sup>		0.180	0.986	
Leiomyoma size				
<4 cm	9	1.04 (0.81–2.17)	1.24 (0.97–2.19)	0.237
4–<6 cm	27	1.72 (1.20–1.97)	1.91 (1.57–2.42)	0.026
6–<8 cm	12	1.57 (1.29–1.97)	1.91 (1.69–2.70)	0.009
8–<10 cm	10	1.64 (1.46–2.30)	2.20 (2.04–2.97)	0.237
≥10 cm	7	1.28 (1.29–2.23)	1.97 (1.91–2.30)	0.273
P <sup>b</sup>		0.747	0.368	
Leiomyoma type				
Multiple	55	1.56 (1.14–1.94)	1.90 (1.57–2.22)	0.000
Single	10	1.74 (1.23–2.88)	2.53 (1.77–3.50)	0.114
P <sup>b</sup>		0.152	0.031	
Leiomyoma location				
Submucous	8	1.82 (1.65–1.95)	2.40 (1.59–2.61)	0.273
Intramural	45	1.56 (1.15–2.02)	1.94 (1.61–2.50)	0.000
Subserosal	12	1.51 (1.14–2.60)	1.84 (1.51–2.08)	0.575
P <sup>b</sup>		0.553	0.415	
P <sup>a</sup> differences between uterine leiomyoma and adjacent normal myometrium, P <sup>b</sup> differences among different clinicopathological characteristics, in uterine leiomyoma or in myometrium				

leiomyomas ( $r_s=-0.305$ ,  $P=0.026$ ). No correlation was observed between protein levels of 14-3-3 gamma and ER-beta ( $r_s=-0.036$ ,  $P=0.796$ ) or PR ( $r_s=-0.157$ ,  $P=0.262$ ).

5. DISCUSSION

14-3-3 gamma is an oncogene that is involved in the activation of phosphoinositide 3-kinase and mitogen-activated protein kinase signaling cascades (22, 23). Previous studies showed that 14-3-3 gamma is dynamically regulated by several factors. In lung cancer, 14-3-3 gamma gene is a downstream target of p53, and p53 dysfunction led to its overexpression (24). In hematopoietic cells, the expression of 14-3-3 gamma gene was significantly induced by interleukin-3 at both the transcriptional and translational levels (23).

In recent years, the potential involvement of 14-3-3 gamma protein in several types of cancer has attracted wider attention. Song *et al.* (14) reported that 14-3-3 gamma protein levels in breast cancer were significantly higher than in non-cancerous mammary gland tissues, and that overexpression of 14-3-3 gamma was associated with a larger tumor size and a higher tumor grade. Similarly, 14-3-3 gamma protein levels were significantly higher in cholangiocarcinoma than in normal tissue, although the abundance status was not related to tumor location and size, pathologic differentiation, lymphatic permeation, lymph node metastasis, or tumor stage (25). Likewise, 14-3-3 gamma protein levels were increased in human glioma compared to normal tissues (3). In contrast to cancers, neither human meningioma nor normal tissues were found to express 14-3-3 gamma gene (26).

Using proteomics (15), we have previously found that 14-3-3 gamma protein levels are lower in uterine leiomyoma than in an adjacent normal myometrial tissue. To explore the possible molecular mechanism of decreased 14-3-3 gamma expression in uterine leiomyomas, we analyzed the DNA methylation status of 14-3-3 gamma gene promoter. Transcriptional silencing of CpG islands by DNA hypermethylation in gene promoter regions is known to lead to the inactivation of tumor suppressor genes and previous studies have found that 14-3-3 sigma, an isoform of 14-3-3 gamma, is aberrantly hypermethylated in a variety

**Table 4.** The expression of PR, ER alpha, and ER beta in leiomyoma and myometrium during the menstrual cycle [median (P25–P75)]

	N	Total	Proliferative phase	Secretory phase	Atrophic phase
PR					
Leiomyoma	65	1.27 (0.90–1.95)	1.43 (1.01–2.01)	1.64 (1.25–1.93)	2.41 (1.13–3.37)
Myometrium	65	0.99 (0.56–1.38)	1.12 (0.73–1.39)	0.84 (0.50–1.37)	0.91 (0.56–2.95)
<i>P</i>		0.000	0.001	0.003	0.273
ER alpha					
Leiomyoma	65	1.76 (0.79–2.27)	1.88 (1.07–2.38)	0.97 (0.57–1.78)	2.18 (1.37–2.78)
Myometrium	65	1.13 (0.58–1.46)	1.14 (0.65–1.33)	0.71 (0.36–1.35)	1.90 (0.90–2.94)
<i>P</i>		0.000	0.000	0.053	0.715
ER beta					
Leiomyoma	65	0.97 (0.77–1.16)	0.98 (0.76–1.18)	0.97 (0.85–1.15)	0.83 (0.69–1.13)
Myometrium	65	0.71 (0.60–0.82)	0.73 (0.59–0.82)	0.73 (0.63–0.85)	0.68 (0.56–1.08)
<i>P</i>		0.000	0.000	0.001	0.273

of human cancers. E.g., Kaneuchi *et al.* (27) reported that inactivation of the 14-3-3 sigma gene in ovarian cancer was mediated by DNA hypermethylation. Similarly, Liang *et al.* (3) found that 14-3-3 sigma deficiency in glioma cells was due to hypermethylation of DNA in a coding region of this gene. Indeed, our MSP analysis revealed that methylation frequency of the 14-3-3 gamma gene promoter is higher in uterine leiomyoma than in normal myometrial tissue. Furthermore, this hypermethylation correlated with a decreased 14-3-3 gamma protein level in uterine leiomyoma tissue, particularly in the proliferative and secretory phases of menstrual cycle. This suggests the possibility that hypermethylation of the 14-3-3 gamma gene promoter might affect 14-3-3 gamma gene expression and, directly or indirectly, contribute to a decrease in 14-3-3 gamma protein levels in uterine leiomyomas.

In the present study, we verified our earlier proteomics findings (15) that 14-3-3 gamma protein levels were lower in uterine leiomyoma than in the adjacent normal myometrial tissue. This emphasized the possibility that 14-3-3 gamma may be involved in the development of uterine leiomyomas. Further investigations are needed to explore the role of the down-regulation of 14-3-3 gamma protein levels in the early onset of uterine leiomyoma, and in the transformation of normal myometrium to uterine leiomyoma.

Herein, we also presented a detailed investigation of 14-3-3 gamma protein levels under specific physiological circumstances, such as the menstrual cycle, and tumor clinicopathological characteristics. 14-3-3 gamma protein levels in uterine leiomyoma tissue were not associated with the menstrual cycle phase, patient age, and leiomyoma size, type, and

location. Without needing to consider that the factors like menstrual cycle phase, patient age, and leiomyoma size, type, and location would affect the choice of leiomyoma sample. This observation provides an experimental basis for further studies exploring the role of 14-3-3 gamma in tumorigenesis and growth of uterine leiomyoma, e.g. by overexpressing or knocking down the 14-3-3 gamma gene in primary leiomyoma cell cultures.

In the present study, 14-3-3 gamma protein levels were lower in uterine leiomyomas than in normal myometrium, especially in proliferative and secretory phases of the menstrual cycle, suggesting 14-3-3 gamma is necessary for the growth of normal myometrium and it might have oncogenic potential in uterine leiomyoma. The loss of regulation of 14-3-3 gamma in some aspects in normal myometrium lead to the happen of leiomyoma. This could be explained by the intrinsic biological differences between leiomyoma and normal myometrial tissues. Furthermore, 14-3-3 gamma protein levels in women with leiomyoma sizes of 4≤d<8 cm, with multiple, or intramural leiomyomas, differed between tumor and normal tissues. This suggests that 14-3-3 gamma may play an important role in women of reproductive age, who have large, multiple, and intramural leiomyomas. Our findings also evince the potential of 14-3-3 gamma protein as a biomarker in the prognosis of patients with leiomyomas. One has to interpret these results with caution, however, since control tissues were selected from myometrial tissues adjacent to leiomyomas. It is possible that such myometrial tissue differs from true normal myometrium that comprises both transitional stromal cells and infiltrated neutrophils. We minimized this concern by evaluating myometrial tissues sampled at sites distant from the leiomyoma site.

The occurrence and development of leiomyomas depends partly on estrogen and progesterone. In women of reproductive age, estrogen levels are high in the proliferative phase of the menstrual cycle, whereas progesterone rises in the secretory phase of the cycle (28). In contrast, levels of both estrogen and progesterone decline in postmenopausal women (18). Thus, it has been proposed that the expression of 14-3-3 gamma gene may be regulated by sex hormones. Previous studies have found an association between 14-3-3 proteins and ERs. De Vries-van *et al.* (29) reported that the C-terminal F-domain of ER-alpha contains a mode-III motif for 14-3-3 proteins binding in breast cancer cells, suggesting a functional interaction between these two proteins. In uterine leiomyoma tissue, Wang *et al.* (8) observed a negative correlation between the protein expression of 14-3-3 gamma and ER/PR. Moreover, Nakayama *et al.* (4) found a negative correlation between the expression of 14-3-3 gamma protein and ER-alpha, and PR and the estrogen-responsive finger proteins in endometrial carcinoma. In the present study, we found that in leiomyomas, decreased levels of 14-3-3 gamma protein were associated with increased levels of ER-alpha, but not PR or ER-beta. This hints at a role of an interaction between 14-3-3 gamma and ER-alpha in the tumorigenesis of uterine leiomyomas. Further investigation of this interaction may provide better understanding of the pathophysiological role of 14-3-3 gamma in uterine leiomyomas.

## 6. CONCLUSION

In conclusion, our study revealed that in women in the proliferative and secretory menstrual phases, DNA hypermethylation in the promoter region of the 14-3-3 gamma gene might contribute to its down-regulation in uterine leiomyomas. The uterine leiomyoma 14-3-3 gamma protein levels did not change with menstrual cycle stage and patient age, or the size, location, and type of leiomyoma. The levels of 14-3-3 gamma protein were lower in leiomyomas than in myometrium. This was particularly significant in the proliferative and secretory menstrual phases, and in women younger than 48 years old. This suggests a role for 14-3-3 gamma in the development of leiomyomas, particularly in women of reproductive age, who have large, multiple, and intramural leiomyomas, and affords basis for designing further investigations into the role of 14-3-3 gamma in tumorigenesis and growth of uterine leiomyomas. Furthermore, protein levels of 14-3-3 gamma negatively correlated with ER-alpha levels, indicating a possible interaction between these two proteins. Additional studies are needed to investigate the epigenetic and hormonal regulation of 14-3-3 gamma in leiomyomas, and the precise role in the development of uterine malignancies prevalent in women of reproductive age.

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**Abbreviations:** ER: estrogen receptor; PR: progesterone receptor; MSP: methylation specific polymerase chain reaction

**Key Words:** 14-3-3 Gamma, Uterine Leiomyoma, Menstrual Cycle, Methylation

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