Uterine leiomyomas (fibroids) are the most common neoplasms in women of reproductive age. The lifetime incidence of this disease is about 70% and 80% in White and Black women, respectively (1). Despite the high incidence of fibroids in the general population, about 20% of women with fibroids experience clinical symptoms, resulting in 200,000 hysterectomies in the United States annually (2). Severe symptoms such as menometrorrhagia, anemia, dysmenorrhea, pelvic pain, sexual dysfunction, reproductive failure, and compression of adjacent pelvic viscera are often induced by a submucosal location, rapidly growing large leiomyomas, as well as, a large number of tumors in a single uterus.

Currently, many large and symptomatic fibroids are treated surgically, as no effective long-term medical management is available. In the past several years, significant progress has been made in the identification of molecular and genetic changes involving in the pathogenesis of fibroid growth. These include the characterization of the genes and signal pathways involving in the sex steroid hormone, mitogenic and growth factors, and extracellular matrix, which are associated with fibroid growth (3–6). All such studies help to identify the potential molecular targets for the treatment of rapidly growing fibroids.

About 80% of women with fibroids are clinically asymptomatic (mostly related to small and slow growing fibroids or subserosal location). It is interesting as to why such a large numbers of fibroids are clinically quiescent or biologically inactive. It has been observed that in the same sex steroid hormone status (e.g., multiple leiomyomas from a single uterus), the natural growth rate of fibroids widely varies; some grow quickly, whereas others remain small (7). Davis et al. (8) examined the natural growth rate of fibroids in vivo in women who were symptomatic and asymptomatic, and they had similar findings as did DeWaay et al. (7). In addition, both studies found that larger leiomyomas had a tendency to grow faster than small ones (7, 8). Although the difference of growth rate between large and small leiomyomas remains to be determined, a proportion of fibroids seem to lose the potential to grow. Although much attention is paid to why fibroids grow, it will be interesting in searching for the molecular and cellular reasons why some fibroids remain small or inactive.
In this study, we pursue a different approach to investigate whether a cellular senescence is a molecular and cellular basis for inactive fibroids.

**MATERIALS AND METHODS**

**Patients and Tissue Samples**

This study included 86 fibroids from 14 symptomatic patients of pre- or perimenopause. All clinical information was well documented. Of all patients, five were Black women, five were White women, and four were other races/ethnicities. Patient age at surgery, number of tumors, endometrial phase, and procedure type are summarized in Table 1. Tumor sizes ranged from 1 to 10 cm (mean 3.6 ± 0.41 cm). Matched myometrium were also collected if available. The study was approved by our institutional research board.

Tissue samples were collected within 1 to 4 hours after surgery. Fresh tumor tissue and matched myometrium were collected as tissue core samples measuring 0.4 cm in diameter and 1.0 cm in length. All tumor sections were taken in the region next to peripheral of tumors larger than 2 cm (0.5–1.0 cm away from peripheral). The core was subsequently bisected and half frozen in OCT medium, and the other half was submitted and prepared to be formalin fixed and paraffin embedded (FFPE).

**RNA Preparation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

Total RNA isolation was performed using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer’s instructions. For the detection of mature microRNAs, mirVana qRT-PCR Primers and the mirVana qRT-PCR Detection Kits (Ambion) were used and optimized according to the abundance of microRNAs in the samples. In brief, 50 to 100 ng of total RNA were reverse transcribed with specific microRNA primers. A total of 15 to 30 cycles were performed for quantitation. U6 was used as control for the quality of RNA.

**Senescence-Associated β-Galactosidase (SA-β-Gal) Activity**

The SA-β-Gal stain is a well-established and reliable technique in detecting cells of senescence (9). We have tested and used this modified stain for primary culture leiomyoma cells (Fig. 1) and fresh collected tumor tissue sections (Fig. 1) under the guidance of Dr. Campisi (10). In brief, cells cultured on tissue culture chamber slides (Cole-Parmer, Vernon Hills, IL) and fresh-frozen tissue sections on glass slides were washed twice with phosphate-buffered saline (PBS), fixed with 2% formaldehyde + 0.2% glutaraldehyde in PBS for 3 to 5 minutes at room temperature, and washed an additional two to three times with PBS. Slides were then incubated with the SA-β-Gal stain solution (9) at 37°C without CO2 for 12 to 16 hours. Slides were counterstained with Eosin. Senescence was identified as a blue stain and quantified as a percentage.

**Let-7c microRNA In Situ Hybridization**

The hybridization system and probes, miRCURY LNA, Let-7c, and U6, were purchased from Exiqon (Vedbaek, Denmark). The detailed procedure for miRNA in situ hybridization (MISH) was followed as per manufacturer’s protocol (11). In brief, 4-μm slides were prepared. Following brief fixation, the slides were prehybridized with 1× hybridization buffer without probe. The hybridization was performed overnight in a 1× hybridization buffer (30–70 μL) with predenatured miRCURY LNA, let-7c, or U6 probes. After washing, the slides were blocked and incubated with AP conjugated anti-DIG Fab fragments (1:1500, Roche, Indianapolis, IN) and visualized for color detection.

**Immunomarkers and Immunohistochemistry**

Antibodies used for the study included HMGA1 (kindly provided by Dr. E. Hernando), HMGA2 (BioChem Inc., Hawaiian Gardens, CA), Ki-67 and p16INK4a (Ventana Medical System Inc., Tucson, AZ, and Neomarkers, Fremont, CA). The tissue was sectioned at 4 microns. After deparaffinization and antigen retrieval, all immunohistochemical staining was performed on a Ventana Nexus automated system.

**Data and Statistical Analysis**

The SA-β-Gal stain and Ki-67 positive cells were scored quantitatively as a percentage of total cells. The levels of let-7 miRNA expression detected by RT-PCR and MISH were scored quantitatively by density photometry (Scion image/NIH free ware). Immunoreactivity for HMGA1, HMGA2, and p16INK4a were scored semiquantitatively using a combined immunointensity and percentage scores. Paired t test and χ² analysis were used for statistical significance. A value of P < .05 was considered significant (Table 1).

**RESULTS**

**Senescence in Fibroids**

Fibroids collected for the study were from patients who had symptomatic fibroid uteri and underwent either hysterectomy or myomectomy. For myomectomy, all fibroids were from intramural or subserosal location. For hysterectomy, only three fibroids were found to be submucosal location and all others were from intramural or subserosal location (Table 1).

The SA-β-Gal stain for senescent cells is a well-established technique and highlights cells that have undergone irreversible rest in G0 and permanent loss of cell division capability. To determine whether SA-β-Gal stain can be used to identify senescence in uterine fibroids and normal myometrium, we first examined the SA-β-Gal stain in primary culture of fibroid and myometrial cells. The cultured cells were stained at two time points: 3 to 5 days after initial primary cell culture, and 6 to 8 weeks after primary cell culture was maintained with three passages, at which, most of the cells have undergone senescence. As illustrated in Figure 1A, there was almost no SA-β-Gal stain in the cells
cultured for 3 to 5 days. In contrast, most of leiomyoma cells were SA-β-Gal stain positive following 6 to 8 weeks of culture.

We then used the SA-β-Gal stain techniques established in primary cell culture for tumor sections prepared from fresh frozen tissue (see Materials and Methods). Photomicrographs illustrate the various types of tumor cell senescence (SA-β-Gal stain) with a count stain of eosin in a fresh-frozen section of fibroids. (A1) Monolayer of leiomyoma cells of 5 days following initiation of the primary culture. (A2) Leiomyoma cells stained by SA-β-Gal following 6 weeks of primary culture with three passages. Blue color is identified as SA-β-Gal positive (see Materials and Methods). (B1–4) Photomicrographs illustrate the various types of tumor cell senescence (SA-β-Gal stain) with a count stain of eosin in a fresh-frozen section of fibroids. (B1) Chimerical type; (B2) patchy and clustered type; (B3) localized type; and (B4) diffuse type. Yellow bars represent 200 μm.

There were several different patterns of tumor senescence in fibroids. Type 1: chimerical type: senescent and nonsenescent tumor cells are intermingled (Fig. 1B1). This type of senescence seems to be early stage of tumor cell senescence.

SA-β-Gal stain is reported as a percentage. We defined three levels of senescence: [1] low or nonsenescent group (demonstrating <10% of tumor cells staining), accounting for 42% (34 of 82) of tumors; [2] intermediate senescent (10%–40% of tumor cells staining), accounting for 47% (39 of 82) of tumors; and [3] a high senescent group (>50% of tumor cells staining), accounting for 11% (9 of 82) of tumors.
Type 2: patchy type: senescence was either clustered or found as aggregates within the tumor (Fig. 1B2). They were easily recognized by SA-β-Gal stain, and may be the second stage of senescence following the chimera stage. Type 3: localized and large scale of senescence: senescence was identified in large islands of senescence (Fig. 1B3). Type 4: complete senescence of entire tumor (Fig. 1B4). This is the end stage of fibroids with no growth potential.

To evaluate the association of senescence with patient clinical characteristics, we compared patients’ age and tumor size to the level of senescence. Senescence positively correlated with age (Fig. 2). For patients 44 years of age and under, the average percentage of senescence was about 15%. In comparison, patients 45 and above, the average levels of senescence was about 25%. These differences in senescence with respect to age was statistically significant (P < .05) (Fig. 2C). Further, we compared the level of senescence with tumor size. Smaller tumors demonstrated a larger percentage of senescence; however, these differences were not statistically significant (P = .24; Fig. 2B).

In patients with multiple fibroids, no differences of senescence among different patients were noted. The levels of senescent fibroids seemed to be randomly distributed in this group of patients. To avoid regional effect on senescence, we collected tumor tissues in defined region (next to peripheral, see Materials and Methods), particularly for those large fibroids.

Among 86 fibroids, 33 fibroids were randomly selected to evaluate the proliferative index by quantifying the immunoreactivity for Ki-67. The Ki-67 index was determined by identifying the percentage of immunopositive cells in each tumor. This revealed that tumor senescence was inversely correlated with the Ki-67 index (r = −0.59) (Fig. 2D). These findings support that senescence, detected by SA-β-Gal stain, reflects the inactivity of a subtype of fibroids.

Senescence and let-7 Expression

In our previous study, we found that the level of the let-7 miRNA family was significantly higher in small fibroids in comparison to large ones (12, 13). Furthermore, let-7 was found to target several cell cycle genes, such as CDK6, CDC25a, and CCND2 (14). To determine whether let-7 miRNA expression was associated with senescence in fibroids, we evaluate the levels of let-7 miRNAs in tumors expressing high levels and low levels of senescence. Briefly, we selected seven highly senescent and seven nonsenescent fibroids for let-7 expression. By semiquantitative RT-PCR, we determined the expression levels of three let-7 family members, namely, let-7c, d, and f-2 (which were identified to demonstrate the most aberrant expression in fibroids) (13) (Fig. 3A). Interestingly, there was a significant difference in the expression of let7 miRNAs (P < .05) (Fig. 3B).

To further validate these findings, we conducted MISH in 65 out of 86 fibroids; MISH was performed in fresh-frozen
Senescence and Other Associated Gene Expression

Senescence is a complex molecular mechanism that involves many signaling pathways. Among the many stimuli that can induce senescence, the RAS trigger p53-dependent p16INK4a is well-characterized both in vivo and in vitro (15). In addition, using RAS to induce senescence in fibroblasts in vitro has been shown to cause an overexpression of HMGA1/2 (16). To examine the role of p16INK4a and HMGA1/2 in fibroid senescence, we performed immunohistochemical studies for the targets p16INK4a and HMGA1/2 in 56 fibroids of which the senescence status was well documented. In these 56 fibroids, six were immunopositive for HMGA1 (11%) and seven were immunopositive for HMGA2 (13%). No correlation of HMGA1/2 and the levels of senescence were identified. These findings could result from the low rate of HMGA1/2 positivity or HMGA1/2 possibly has no role in the induction of senescence in fibroids.

We then examined p16INK4a, one of key molecules in senescence. In general, a large proportion of fibroids showed varied immunoreactivity for p16INK4a. Of significance, one fibroid, (100% senescence) showed a strong and diffuse immunoreactivity for p16INK4a, a high level of let-7c, and a low level Ki-67.

DISCUSSION

Fibroids, in vivo, are often multiple, and interestingly, do not grow at equal rates. In fact, some tumors are shown to not grow at all (7, 8). Our study addresses whether senescence plays a role in preventing fibroid growth. Among a total of 86 “symptomatic” fibroids (tumor sizes ranged from 1–10 cm), 11% of them were highly senescent (50% of tumor cells with SA-α-Gal; Fig. 2A), 47% were intermediately

tissue section (Fig. 3C). The level of let-7c was normalized by U6 in all tumors. The intensity of let-7c expression was scored by density photometry (see Materials and Methods). A correlation analysis showed a moderate correlation between senescence and let-7c expression (r = .44) (Fig. 3D).
senescent (10%-40% of SA-α-Gal), and approximately 40% of tumors were low or non-senescent (<10% of SA-α-Gal) (Fig. 2A). When we compared the levels of senescence with tumor size, there was a tendency of higher levels of senescence in small tumors and lower levels in large tumors (Fig. 2B). Given our previous findings of high let-7s in small fibroids (12, 13) and a negative regulation of some cell cycle genes by let-7s (14), we further examined mature let-7 c, d, and f-2. We found an increase of let-7 expression in highly senescent fibroids (Fig. 3). These observations suggested that let-7s might be related to tumor senescence. The recent studies identified that let-7s can be one of important molecules in regulation of cell cycle related gene functions (14), and therefore, let-7 repression of cell cycle genes makes it a plausible candidate responsible for the induction of senescence in fibroids.

Senescence depends on a number of pathways, and together, these pathways result in a permanent and irreversible cell-cycle blockade. Cell cycle progression is mainly controlled by the activity of a family of protein kinases known as the cyclin-dependent kinases (CDKs) (17). The function of these CDKs is carefully regulated. There is a group of small proteins known as the CDK inhibitors (CKIs). Among these CKIs, p16INK4a has been shown to be an important player in the induction of senescence (17). In this study, we were not able to establish the relationship between senescence and p16INK4a expression. Recent gene profile analysis showed a significant difference of p16INK4a expression amongst benign and malignant uterine smooth muscle tumors (18). p16INK4a is significantly overexpressed in malignant uterine smooth muscle tumors. This finding is further supported by several immunohistochemistry studies, in which the differential expression of p16INK4a gene products among benign, atypical, and malignant uterine smooth muscle tumors are well documented (19–22). Although dysregulation of p16INK4a in uterine leiomyosarcomas is not known, it is less likely to be associated with senescence. It is generally believed that there are different pathways of tumorigenesis in leiomyoma and leiomyosarcoma, and the role of p16INK4a in leiomyoma deserves a further investigation.

MiR-34 is a microRNA that can promote senescent-like changes through repression of CDK genes in vitro (23). We found miR-34 is overexpressed in fibroids (13). The role of miR-34 in association with senescence in fibroids requires a further evaluation. Senescence can be an important cellular mechanism in fibroids. The current study identified that about 60% of symptomatic fibroids were either partially or completely senescent. We can speculate that the rate and degree of senescence in those women who had small and asymptomatic fibroids will be much higher than that in symptomatic women. Therefore, senescence could be one of important evolutionary mechanisms in preventing fibroid growth and the development of symptoms. Further, we postulate that once a uterine smooth muscle cell is transformed into a leiomyoma, the tumor will face one of two fates. The first fate is that the tumor will gain additional genetic alterations that promote tumor growth leading to symptomatic disease. The second fate is senescence, triggered by molecular and environmental stresses. Further study into how and what molecular and environmental factors promote fibroid senescence will both broaden our understanding of this unique process and provide novel therapeutics for this common disease.

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REFERENCES