Epidermal growth factor–containing fibulin-like extracellular matrix protein 1 expression and regulation in uterine leiomyoma

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Objective: To determine the presence, differential expression, and regulation of epidermal growth factor–containing fibulin-like extracellular matrix protein 1 (EFEMP1) in uterine leiomyomas.
Design: Laboratory in vivo and in vitro study with the use of human leiomyoma and myometrial tissue and primary cells.
Setting: Academic medical center.
Sample(s): Leiomyoma and myometrial tissue samples and cultured cells.
Intervention(s): 5-Aza-2′-deoxycytidine (5-aza-dC) treatment.
Main Outcome Measure(s): Fold-change difference between EFEMP1 and fibulin-3 expression in leiomyoma tissue and cells compared with matched myometrial samples, and fold-change difference in EFEMP1 expression with 5-Aza-dC treatment.
Result(s): In vivo, EFEMP1 expression was 3.19-fold higher in myometrial tissue than in leiomyoma tissue. EFEMP1 expression in vitro was 5.03-fold higher in myometrial cells than in leiomyoma cells. Western blot and immunohistochemistry staining of tissue and cells confirmed similar findings in protein expression. Treatment of leiomyoma cells with 5-Aza-dC resulted in increased expression of EFEMP1 in vitro.
Conclusion(s): The EFEMP1 gene and its protein product, fibulin-3, are both significantly down-regulated in leiomyoma compared with myometrium when studied both in vivo and in vitro. The increase in EFEMP1 expression in leiomyoma cells with 5-Aza-dC treatment suggest that differential methylation is responsible, in part, for the differences seen in gene expression. (Fertil Steril® 2016;105: 1070–5. ©2016 by American Society for Reproductive Medicine.)
Key Words: leiomyoma, EFEMP1, fibulins, extracellular matrix

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Uterine leiomyomas or "fibroids" are benign sex steroid–sensitive smooth muscle tumors of the uterus. Leiomyomas occur in as many as 30%–50% of reproductive-age women and have an overall cumulative incidence of 70% by the age of 50 years, making them the most common benign tumors in women (1). Symptoms associated with leiomyomas include abnormal uterine bleeding, pelvic pain or pressure, increased abdominal girth, and recurrent pregnancy loss, all of which reduce quality of life (2, 3). These symptoms are why leiomyomas remain the leading cause of hysterectomy in the United States and cost the U.S. health system an estimated $34.4 billion annually (4). Despite their prevalence and public health impact, the etiology of leiomyomas remains unclear.

What we do know is that the extracellular matrix (ECM) of leiomyomas is much more extensive than adjacent myometrium and has been shown to be quantitatively and

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qualitatively different. Although collagens have been well studied in leiomyomas and myometrium, fibulins as a class have not been well characterized. Fibulins are a class of seven secreted extracellular glycoproteins, characterized by tandem calcium-binding epidermal growth factor (EGF)–like domains and a unique C-terminal fibulin structure (5). As a whole, fibulins have been widely studied for their involvement in tumor biology and are specifically implicated for their roles in cell morphology, growth, adhesion, and motility (6). Fibulin-3 is encoded by the gene EFEMP1 (epidermal growth factor–containing fibulin-like extracellular matrix protein 1) and has been suggested to have paradoxic effects on tumor biology. Some evidence suggests that fibulin-3 may act as an antagonist of angiogenesis (7), supporting studies that demonstrate that EFEMP1 down-regulation and therefore a loss of fibulin-3 protein may be associated with increased tumor angiogenesis in several cancers (8–11). However, other studies have shown that increased expression of EFEMP1/fibulin-3 may promote tumor growth in pancreatic adenocarcinoma (12) and cervical cancer (13).

To our knowledge, neither the presence nor expression pattern of EFEMP1 or fibulin-3 has been formally investigated in leiomyomas. In the present study, we explored the expression and regulation of EFEMP1 and fibulin-3 in leiomyomas and myometrium. We hypothesized that similarly to other solid tumors, EFEMP1 is differentially expressed in leiomyomas versus myometrium, both in vivo and in vitro.

MATERIALS AND METHODS

Study Subjects

Leiomyoma and matched myometrial tissues were collected from subjects (n = 20) undergoing hysterectomy for uterine leiomyomata at Northwestern Memorial Hospital. The age range of the subjects was 35–52 years (mean 44.5 ± 4.69 y). All subjects were premenopausal nonsmokers, and none were taking hormonal medication within 3 months of surgery. Written informed consent was obtained from every subject. The study protocol was approved by the Institutional Review Board at Northwestern University.

Tissue Specimens

Tissue samples were taken from the operating room to the pathology department within 30 minutes of being removed from the patient. Resected leiomyomata ranged in size from 3.5 cm to 12 cm in diameter and were both subserosal and intramural in nature. The pathologist was provided tissue samples as follows. Leiomyoma samples were obtained within 1–2 cm of the outer capsule of the leiomyoma. Myometrial samples were obtained within 2 cm of the resected leiomyoma. The tissues were rinsed three times in cold phosphate-buffered saline solution (PBS), and transported back to the lab in DMEM/F-12 medium on ice. The tissues were then processed for cell culture, paraffin embedding, flash-freezing, protein isolation, or nucleic acid isolation as described below.

Nucleic Acid Isolation

Leiomyoma and myometrial tissue samples were kept in RNA-later, (Invitrogen), stored at −20°C, and, when needed, homogenized in RLT buffer (Qiagen) with the use of a Polytron PT 2100 homogenizer (Brinkmann Instruments). Insoluble material was then removed by means of centrifugation at 5,000 rpm for 10 minutes. The supernate was harvested and total RNA extracted with the use of the RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were later confirmed by spectrophotometry with the use of Nanodrop ND-1000. RNA integrity was confirmed with the use of gel electrophoresis.

Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was used to amplify the mRNA expression of EFEMP1 by means of the ABI Taqman gene expression system and the ABI7900 sequence detection system (Applied Biosystems). The mRNA expression levels are reported as fold change, as calculated with the use of the $2^{-\Delta\Delta Ct}$ methodology (14).

Cell Cultures

The procedure for isolation of primary cells from leiomyomas and myometrium has been previously described (15). Briefly, fresh leiomyoma and myometrial tissues were rinsed in PBS, cut into 2–3-mm cubed pieces and incubated with collagenase and DNase for 8–10 hours in a warm shaking incubator. The digested lysate was then strained to remove any undigested tissue and debris. The lysate containing the cells was then centrifuged for 10 minutes at 5,000 rpm at room temperature. The resulting cell pellet was rinsed three times with DMEM/F-12 medium containing 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic mixture. The cells were counted and plated at the desired concentration in the same media. Once the plates were confluent, subcultures of each type of tissue were trypsinized and passaged up to two times with 0.1% trypsin–.5% EDTA.

5-Aza-2′-deoxycytidine (Decitabine) Treatments

Primary cells were treated with 5-aza-dC, an epigenetic modifier that inhibits DNA methyltransferase activity. Primary leiomyoma cells were placed in serum-free DMEM/F-12 medium overnight and then treated with varying concentrations of 5-aza-dC (0, 1, 5, 10, and 20 μmol/L) for 5 days. All 5-aza-dC–containing media were changed daily. After 5 days, RNA and protein were isolated.

Protein Isolation and Western Blot

For protein extraction from tissue, flash-frozen leiomyoma and myometrial tissues were removed from the −80°C freezer, ground in liquid nitrogen, and lysed in T-PER protein extraction reagent (Thermo Fischer Scientific). For protein isolation from primary cultured cells, primary cells were washed in ice-cold PBS and suspended in T-PER. The protein was extracted following the manufacturer's protocol and the concentration quantified with the use of the bicinchoninic (BCA) assay