SPANDIDOS PUBLICATIONS

SPARC-like1 mRNA is overexpressed in human uterine leiomyoma

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Abstract. Uterine leiomyomas (ULs), also known as fibroids, are benign and monoclonal tumors frequently found in the female population. The genetic alterations that contribute to UL tumor development have not been well established, and the goal of this study was to reveal gene expression variation between ULs and healthy uterine tissue. We compared the gene expression profiles of 13 UL tumors with that of their adjacent normal tissue using the Differential Display mRNA assay (DDRT-PCR). Among the genes upregulated in some of the UL samples, several genes previously described in the context of cancer were identified, namely *LIMK1*, *MCM3* and *UHRF1*. In addition, we identified a cDNA present in UL samples from distinct patients, which was absent from their normal tissue. Direct sequencing of this cDNA revealed a human *SPARC-like1 (SPARCL1)* mRNA homology. Through semi-quantitative PCR, we demonstrated that *SPARCL1* was upregulated in approximately 77% of UL samples, but was absent in normal tissue. Real-time PCR (QPCR) revealed that *SPARCL1* expression was increased 5-fold in ULs compared to adjacent normal tissue. These results suggest that the *SPARCL1* gene is involved in UL development.

Introduction

Uterine leiomyomas (ULs), also called uterine fibroids, are the most common benign tumors of smooth muscle origin. Although many women are asymptomatic and thus unaware of their condition, others have aggravating symptoms, such as chronic pelvic pain, dysfunctional uterine bleeding and anemia, which require treatment. Usually, multiple polyps on the uterus of the same patient represent ULs where each polyp has an independent genetic origin (1). ULs are clinically apparent in approximately 25% of women of reproductive age, with data suggesting that more than 75% of women present at least one asymptomatic myoma during their lifetime. These tumors constitute a significant reproductive health problem and decrease the health-related quality of many women's lives (2).

Several studies have examined the potential causes of UL, including environmental influences, genetics and growth factors. These factors included a previous history of pre-eclampsia, primiparity, obesity, family history of preeclampsia, multiple pregnancies, and chronic medical conditions such as long-term hypertension or diabetes (3). The occurrence of clinically significant fibroids peaks in the proximal menopausal years and declines after menopause, suggesting that steroid ovarian hormones, such as estrogen and progesterone, could play an important role in the growth or reduction of these tumors. In accordance with this hypothesis, gonadotrophin-releasing hormone analogue (GnRH-a) treatment has been demonstrated to have a significant influence on the reduction of ULs (4).

Although the majority of ULs are cytogenetically normal, approximately 40% display cytogenetic alterations frequently involving chromosome 12. A recent study found alterations in the gene expression profile of ULs. HMGA2, a member of the high-mobility-group gene frequently aberrantly expressed in fibroids, maps at 12q15, a region often rearranged at t(12;14) and commonly observed in ULs (5).

Gene expression studies have provided clues elucidating the pathways of leiomyoma development. Weston *et al* found that these fibroids have an anti-angiogenic expression profile (6), which may explain the reduced microvascular density observed in fibroids relative to the myometrium. Furthermore, a review of gene expression data revealed concordant changes in the genes regulating retinoid synthesis, insulin-like growth factor metabolism, transforming growth factor signaling and extracellular matrix formation (7). A larger scale gene expression analysis also contributed to a better understanding of UL (8). However, the biology of UL remains obscure.

The aim of this study was to identify genes with differential expression in ULs and their adjacent normal myometrium. In order to analyze these differences, we compared healthy and neoplastic samples obtained from the same patient. The mRNA Differential Display (DD) technique and RT-PCR were applied to identify alterations in mRNA levels. Among the differentially-expressed genes, the *SPARC-like1 (SPARCL1)* gene, a member of the *SPARC* (*secreted protein acidic and*

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Table I. Upregulated cDNAs in uterine leiomyoma.

Patient	Gene bank homology	Accession no.	Homology (%)	Primers
A	Bicarbonate transporter (SLC4A7), mRNA	NM_003615	93	A5
A	Zinc finger protein 394 (ZNF394), mRNA	NM_032164	95	A5
B	Nerve injury-induced gene 1 (NINJ1), mRNA	NM_004148	100	A5
B	Lim domain-kinase 1 (LIMK1), mRNA	NM_002314	100	A5
B, D, E	SPARC-Like 1 (MAST9, HEVIN), mRNA	NM_004684	100, 98, 99	G7
B, D	Caldesmon 1 (CALD1), mRNA	NM_033157	89, 95	G7
C	Minichromosome maintenance deficient 3 associated protein (MCM3AP), mRNA	NM_003906	91	A5
C	Eukaryotic translation initiation factor 4E (EIF4E), mRNA	NM_001968	95	G7
D	Ribosomal protein L10a (RPL10A), mRNA	NM_007104	96	A5
D	Ubiquitin-like protein containing PHD and RING FINGER domains 1 (UHRF1), mRNA	NM_001048201	97	G7
E	Cytochrome b-245, ß polypeptide (CYBB), mRNA	NM_000397	95	A5
E	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 10 (PSMD10), mRNA	NM_002814	93	C3
E	Period homolog 3 (PER3), mRNA	NM_016831	95	C3
E	Secreted phosphoprotein 2, 24 kDa (SPP24), mRNA	NM_006944	94	C3

Gene bank homology, accession no. and percent homology were obtained by BLAST at www.ncbi.nlm.nih.gov/BLAST. Combination 12-mer primers, which were used to obtain cDNA A with 5, G with 7 and C3, were from GenHunter.

rich in cysteine) protein family, was of great significance as it was found overexpressed in the analyzed UL samples.

Materials and methods

Tissue samples. Normal myometrial and leiomyoma tissues were obtained in pairs from the uteri of patients with symptomatic UL. The samples were surgically removed in a total abdominal hysterectomy performed in accordance with locally-approved regulations at the Fernandes Figueira Institute, FIOCRUZ, Brazil. All patients provided their written consent. From the analyzed cohort, we collected a variable number of leiomyomas and only one normal myometrial sample from the uterus of each patient. All tissue samples extracted measured ~3 cm (±1 cm). Normal myometrial samples were obtained from adjacent tissue within at least 2 cm of the UL polyps. The samples were immediately placed in liquid nitrogen and stored at -70˚C until processing.

RNA extraction and cDNA synthesis. Total RNA from UL and normal myometrial tissue was extracted with TRIzol LS reagent (Invitrogen) according to the manufacturer's protocol and stored at -70˚C. RNA was treated with 1 U DNase I (Invitrogen) for 15 min at 27˚C and inactivated by incubation at 65˚C for 10 min. Integrity was analyzed on RNA formaldehyde/agarose gel. A total of 250 ng of each RNA was reverse transcribed with 200 U MMLV reverse transcriptase (Promega) in the presence of 0.1 μM of each 3'-anchored oligo primer (HT11N, N=A, C and G, equal mole; GeneHunter) in 1X First-Strand RT buffer, 0.5 mM dNTP Mix, 10 mM DTT, 40 U RNaseOUT (Invitrogen) in a 20 μl reaction volume.

mRNA Differential Display. mRNA DD-PCR was performed with a modification to the procedure described by Liang and Pardee (9). Briefly, PCR-radioactive amplification was conducted using 4 μl of cDNA primed with mixed anchored primers (HT11N, N=A, C and G, equal mole; GeneHunter) and a 12-mer arbitrary primer (AP1 to AP8; GenHunter). The cycle parameters were 35 cycles at 95˚C for 20 sec, 40˚C for 2 min and 72˚C for 1 min. A final extension was carried out at 72˚C for 5 min. The resultant products were run on 6% denaturing polyacrylamide gel electrophoresis and analyzed by autoradiogram exposure (O-MAT, Kodak). Identified bands were excised from the gel and cDNA was recovered by incubation at 100˚C in 300 μl water for 10 min, then precipitated and recovered in 10 μl sterile distilled water.

Automatic sequencing. cDNA sequences were obtained by Automatic Sequencing (Applied Biosystems) using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequence spectrums were visualized in the BigDye® Scan Analyzer (Applied Biosystems) and submitted to a database search at the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/blast).

Semi-quantitative RT-PCR of SPARC-like1. Primers were designed according to the sequence of *SPARCL1* mRNA (NM_004684): sense 5'-GCCTGGAGAGCACCAAGAG GCC-3' and antisense 5'-ATGGTCCCCAGCCAAAAGC CTC-3'. One microgram of total RNA was used with the SuperScript III One-Step RT-PCR System and Platinum® Taq DNA Polymerase (Invitrogen). RT-PCR was performed as follows: 1 cycle at 50˚C for 30 min, 94˚C for 2 min, and 25 cycles of 30 sec at 94˚C, 30 sec at 60˚C and 40 sec at 72˚C, with a final cycle at 72˚C for 10 min. PCR product was analyzed by electrophoresis on 1.5% agarose gel. GAPDH was used as a mass control: forward primer 5'-ACCACAGTCCATGCCAT CAC-3' and reverse 5'-CCACCACCCTGTTGCTGTA-3'.

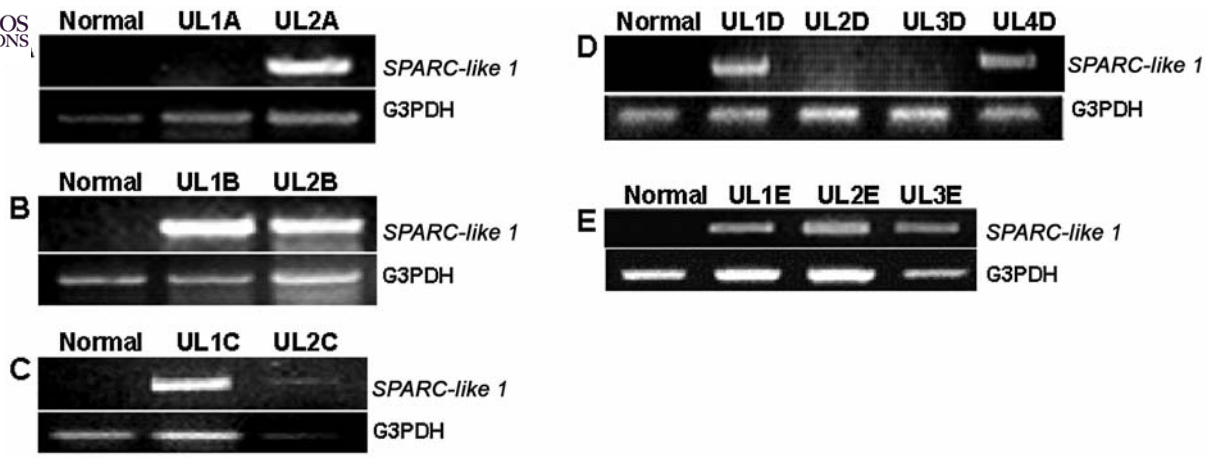

Figure 1. Semi-quantitative RT-PCR analysis of *SPARC-like1* expression in uterine leiomyomas (ULs). More than one UL tissue sample was obtained from patients, represented by A, B, C, D and E. *SPARCL1* was not expressed in normal endometrial samples, whereas 10 of 13 UL samples overexpressed this gene.

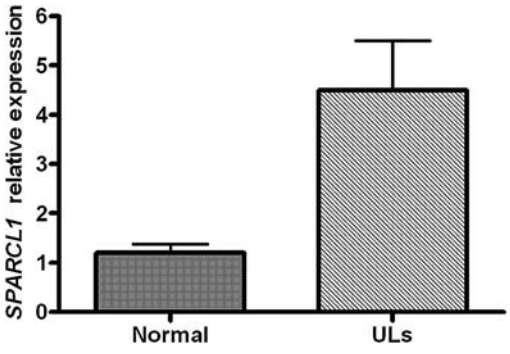

Figure 2. QPCR analysis of *SPARC-like1 (SPARCL1)* gene expression in normal versus uterine leiomyoma (UL) samples. RNA was prepared and analyzed by QPCR. *SPARCL1* expression was increased ~5-fold in the ULs compared to the normal samples. The results represent the mean ± SE for independent cDNA analysis sets normalized to *GAPDH* expression. Data were analyzed by ANOVA ($P<0.05$).

Quantitative PCR. Quantitative PCR analyses were performed with 5 ng total cDNA, 0.5 μM of each of the *SPARCL1* primers and 1X of Power SYBR-Green PCR Master Mix (Applied Biosystems). GAPDH was amplified as an internal standard. PCR protocol was 50˚C for 2 min and 95˚C for 10 min, then 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. All primers demonstrated equal amplification efficiency and specific PCR product through Dissociation Curve analysis. Fold-expression was calculated using the DDCt method. Reactions and analyses were performed using the ABI 7000 PCR and detection system (Applied Biosystems). Data were analyzed statistically by Prisma 4 and ANOVA using the means of the fold expression of all the normal and UL tissues.

Results

Gene expression alterations in leiomyomas. We screened for alterations in the gene expression of leiomyomas using the mRNA DD technique. By comparing leiomyoma and normal myometrium samples from the same patient, we identified several differentially-expressed cDNAs. cDNA fragments positively regulated in the tumor samples of 5 patients were selected for further study. cDNA sequencing and a BLAST (www.ncbi.nlm.nih.gov/blast) search of selected fragments revealed 17 with a high homology to known genes (Table I).

Some of the identified upregulated genes, such as *UHRF1*, *LIMK1*, *PER3* and *ZFP394*, are known transcription factors. Others, such as *CALD1* and *SLC4A7*, are genes involved in smooth muscle physiology. In addition, genes related to cell cycle control, such as *MCM3*, were found to be upregulated. In contrast to these genes, a cDNA fragment with ~350 bp was present in the majority of UL samples and absent in normal tissue. The nucleotide sequence obtained from this cDNA exhibited 100% homology at 320 bp to *SPARCL1* mRNA found in the NCBI human gene database by blast alignment (www.ncbi.nlm.nih.gov/blast).

SPARC-like1 upregulation in uterine leiomyoma. To confirm the high *SPARCL1* mRNA levels, specific primers were designed using the *SPARCL1* gene sequence from the NCBI GenBank (NM_004684) to amplify the gene through RT-PCR. We confirmed the DD results and analyzed a new group of tumor samples as well as their normal counterparts. *SPARCL1* mRNA was undetectable in normal tissue samples (n=5) but present in 77% (10/13) of the analyzed ULs (Fig. 1). These results suggest that the *SPARCL1* gene is involved in the creation or evolution of leiomyomas.

Quantitative PCR analysis. Real-time PCR (QPCR) was used to verify the differences between *SPARCL1* mRNA levels in normal and UL tissue. mRNA levels in the ULs were found to be increased in comparison to those of healthy patient tissue, and expression of the *SPARCL1* gene was 5-fold higher in the ULs than in the normal tissue (Fig. 2).

Discussion

Although ULs are very common in the female population, not much is known regarding the molecular basis of their development. The present study aimed to increase the knowledge of UL pathology by focusing on differences in gene expression. To minimize false-positive results, we compared normal and UL tissue taken from the same patients, thus reducing the chances of findings related to polymorphisms and menstrual cycle phases.

Our results show that several genes, such as *UHRF1*, *LIMK1*, *CALD1*, *MCM3* and others previously correlated with cancer (10-12), were upregulated in a fraction of the analyzed myomas. However, this fraction of overexpressed genes is very small, indicating that although they may contribute to tumor biology, they are not essential to tumorigenic pathology.

In contrast, *SPARCL1* was found present in UL samples from several different patients, but was absent from normal tissue. Using RT-PCR and QPCR, the *SPARCL1* gene was found upregulated in approximately 77% of UL compared to healthy tissue samples. The absence of this gene in three of the UL samples analyzed could be explained by each UL polyp having an independent genetic origin (1), and its upregulation in the majority of UL polyps suggests that the gene is involved, directly or indirectly, in the pathology of UL.

SPARC-like1, also known as HEVIN, MAST9, ECM2, *SPARCL1* and SC1, belongs to the *SPARC* family. This gene was first isolated by the screening of a rat brain expression library against synaptic junction glycoproteins of the central nervous system. Afterward, it was isolated from a human endothelial venule (HEV) cDNA library based on the differential screening of HEV and other vessels (13).

SPARCL1 has been described in many cellular processes, including development, tissue regeneration and cancer, though data concerning it in cancer have been controversial. *SPARCL1* has been found downregulated in many types of cancer, and has therefore been taken as a negative regulator of cell growth and proliferation (13,14). However, *in situ* hybridization has demonstrated the high expression of *SPARCL1* in angiogenic endothelial cells in the stromal areas of invasive pancreatic adenocarcinoma (15). Moreover, *SPARC* genes have been found overexpressed in melanomas (16) and renal carcinoma (17). Additionally, in colorectal carcinoma, where *SPARCL1* was found highly expressed, its upregulation appeared to act as a pro-angiogenic stimulus (18).

SPARCL1 protein has a cysteine-rich region which exhibits a high affinity for copper ions (Cu^{2+}). Regions to which Cu^{2+} binds have in particular been shown to regulate cell proliferation and angiogenesis (13). SPARC protein has been described as a target for cleavage by matrix metalloproteinases (MMPs). For example, MMP-3 cleaves SPARC into active peptides that affect the endothelial cell cycle, migration and angiogenesis (13). Furthermore, SPARC peptides have been linked to the stimulation of angiogenesis (19).

Although the exact role of *SPARC* family members in the physiology and pathology of tumors are not completely clear, several studies suggest that this protein family is involved in tumor maintenance by promoting angiogenesis and, to a lesser extent, cellular proliferation. In this context, our data suggest that the *SPARCL1* gene plays an important role in the pathology of UL by promoting cell proliferation through an angiogenic stimulus.

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