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Gene Expression Profiling of Multiple Leiomyomata Uteri and Matched Normal Tissue from a Single Patient

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Abstract

Objective—To identify differentially expressed genes between fibroid and adjacent normal myometrium in an identical hormonal and genetic background.

Design—Array analysis of 3 leiomyomata and matched adjacent normal myometrium in a single patient.

Setting—University of Colorado Hospital.

Patient(s)—A single female undergoing medically indicated hysterectomy for symptomatic fibroids.

Interventions(s)—mRNA isolation and microarray analysis, reverse-transcriptase polymerase chain reaction, western blotting and immunohistochemistry.

Main Outcome Measure(s)—Changes in mRNA and protein levels in leiomyomata and matched normal myometrium.

Result(s)—Expression of 197 genes was increased and 619 decreased, significantly by at least 2 fold, in leiomyomata relative to normal myometrium. Expression profiles between tumors were similar and normal myometrial samples showed minimal variation. Changes in, and variation of, expression of selected genes were confirmed in additional normal and leiomyoma samples from multiple patients.

Conclusion(s)—Analysis of multiple tumors from a single patient confirmed changes in expression of genes described in previous, apparently disparate, studies and identified novel targets. Gene expression profiles in leiomyomata are consistent with increased activation of mitogenic pathways and inhibition of apoptosis. Down-regulation of genes implicated in invasion and metastasis, of cancers, was observed in fibroids. This expression pattern may underlie the benign nature of uterine leiomyomata and may aid in the differential diagnosis of leiomyosarcoma.

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Keywords

fibroid; microarray; uterine leiomyoma; myometrium; matrix metalloproteinases; fibulins; desmoglein; MST4; PKC β 1; gene expression

INTRODUCTION

Leiomyomata uteri or fibroids are the most common neoplasm of the female genital tract developing primarily during the reproductive years and becoming symptomatic during perimenopause (1,2). Tumors occur in 77% of women, and approximately 25% of Caucasians have clinically significant lesions. The relative risk of fibroids is two to threefold greater in black women than white women and clinical disease is more severe (3–5). Although leiomyomata are benign and rarely result in death, they frequently cause pelvic pain and pressure, dysmenorrhea, menometrorrhagia leading to anemia and less frequently, reproductive dysfunction, including reduced fertility or pregnancy complications, constipation and urinary problems. Leiomyomata are a leading cause of hospitalization for non-pregnancy related gynecologic disorders (6) and are the single most frequent indication for hysterectomy accounting for over 500,000 surgeries per year in the US (7–9). Thus, it is clear that leiomyomata represent a major health problem for women of reproductive age, yet relatively little is known of the etiology or pathophysiology of these tumors.

Previously, in order to identify pathways relevant to development and progression of leiomyomata, several studies have examined the differential gene expression between uterine leiomyoma and normal myometrium using microarray analysis (10–25). However, the different gene array experiments have yielded disparate results, both with respect to those genes identified as being altered in expression and in the direction of the changes (26). This may be a reflection of heterogeneity in myometrial gene expression due to genetic and/or hormonal variations between patients that are independent of the diseased state. In order to avoid the above potential complications we performed a cDNA microarray comparing the gene expression profiles of multiple leiomyomata to matched adjacent normal myometrium from the same patient. Significant changes in gene expression were then validated by reverse transcription PCR, immunohistochemistry or western blotting, and their profiles compared to samples derived from additional distinct patients.

The screening approach of the cDNA microarray and review of the published data for the genes that we identified as differentially expressed enabled us to categorize several targets into specific functional groups. Overall, the results of this study provide very useful insights as to what biological processes may be significantly impacted by gene expression changes in leiomyomata, and the results suggest additional candidate targets for further studies into the mechanisms of pathogenesis.

MATERIALS AND METHODS

Tissue procurement

Portions of leiomyomata, approximately 2 x 3cm sections from the periphery distinct from normal tissue, and matched unaffected myometrium were collected from women (n=11) who were undergoing hysterectomy for indications related to symptomatic leiomyomata. Tissue samples were obtained from each leiomyoma and adjacent matched normal myometrium. The exception was patient BRAD 3 for which only one normal myometrium sample and three leiomyomata samples were collected. None of the patients had received any medical treatment for their fibroids. The tissues were collected at the University of Colorado Hospital with prior approval by the Colorado Multiple Institutional Review Board, under protocol number 03–

642. Immediately after collection in the operating room, a portion of the tissue was snap frozen and stored in liquid nitrogen. Additional portions of tissue were fixed and paraffin embedded for histological evaluation and immunohistochemistry.

RNA isolation

Aliquots (~5 g) frozen tissue sections were pulverized under liquid nitrogen. 50–100 mg of the powdered tissue was placed in 1 ml TRIzol (Invitrogen Life Technologies, Inc., Carlsbad, CA) and was then homogenized with a Polytron probe (Brinkmann Instruments, Westbury, NY). Total cellular RNA was isolated from the tissues and cells using TRIzol per manufacture's instructions. The isolated RNA was quantitated with the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), so that no more than 100 µg was loaded on the column during the DNase treatment. The RNA was DNase treated (twice) using Qiagen RNeasy Mini kit and Qiagen RNase-Free DNase s (Qiagen, Inc.). Quality of purified RNA was assessed on a RNA 6000 Nano Chip using an Agilent 2100 bioanalyzer (Agilent Technologies, Germany).

cDNA synthesis

An aliquot of 1 µg from each total RNA sample was incubated with 2.5 µl of random hexamers in a final volume of 9 µl RNase free water, at 65°C for 5 minutes. The cDNA master mix was made so that the final concentration per reaction tube was 1xPCR buffer, 2.5 mM MgCl₂, 1 mM dNTPs blend, 2 units RNase inhibitor, 5 units MMLV reverse transcriptase. The reaction tube was returned to the PCR machine at 42° C for 60 minutes, 95°C for 5 minutes and samples were stored at 4°C. All RT-PCR reagents were obtained from Applied Biosystems (Foster City, CA).

Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction

All of the primers were obtained from Invitrogen Life Technologies (Carlsbad, CA). Sequences are shown below:

EFEMP1 (Fibulin-3) forward primer: 5'-AGCAGTGACAGGCTCAACTGTGAA-3'

EFEMP1 (Fibulin-3) reverse primer: 5'-CACGAGCACAAGCATTGCACTTAC-3'

MMP-7 forward primer: 5'-GTGGAGTGCCAGATGTTGCAGAAT-3'

MMP-7 reverse primer: 5'-TCCAGCGTTCATCCTCATCGAAGT-3'

MMP-11 forward primer: 5'-CTGCCTCGGAAGAAGTAGATCTTG-3'

MMP-11 reverse primer: 5'-TCTACACCTATCGCTACGCACTGA-3'

MST-4 forward primer: 5'-GGTGGTTCAGCACTGGATCTTCTT -3'

MST-4 reverse primer: 5'-GTGTCCTTCTGCCTTCCATCTCTT -3'

DSG2 forward primer: 5'-CCTAGCCAGCCACAGAGCCTTATT-3'

DSG2 reverse primer: 5'-CGTGGTGTTCCTAGCCGTCATAGA-3'

GAPDH forward primer: 5'-GGCTCTCCAGAACATCATCCCTGC-3'

GAPDH reverse primer: 5'-GGGTGTCGCTGTTGAAGTCAGAGG-3'

Primers were resuspended in 200 µl Rnase free water, quantitated, and diluted at 20 pmoles per µl. Each primer set was tested for optimal cycle number (5 µl aliquots were taken out after 12, 15, 18, 21, 24, 27 cycles) and optimized for the best annealing temperature by using a gradient of 60–70°C. Cycle optimization and temperature optimization was performed on matched normal and leiomyoma samples. Optimal cycle numbers and annealing temperatures were as follows: EFEMP (fibulin-3) 25 cycles at 62 °C; MMP-7 25 cycles at 69 °C; DSG2 27 cycles at 62 °C, MST-4 24 cycles at 62 °C, MMP-11 24 cycles at 63 °C and *GAPDH* for 21 cycles at 65 °C. Master mix for RT-PCR contained 5 µl cDNA, 1xPCR buffer, 2.5mM MgCl₂, 1mM dNTPs blend, 2 µM each of the forward and reverses primers, 1 µl of DNA iTaq polymerase (Bio-Rad) in a total volume of 50 µl. PCR reactions were run on an eppendorf Mastercycler gradient PCR machine at 94°C for 4 minutes followed by the optimized number of cycles (94°C for 30 seconds, optimal annealing temperature between 62–69°C for 45 seconds, 72°C for 45 seconds) followed by a final 72°C for 5 minutes extension time. 8 µl of the RT-PCR products were run on a 2% agarose gels stained with ethidium bromide, along with a 100 bp ladder (New England Biolabs, Ipswich, MA). Alpha Innotech Gel Documentation Imager was used to visualize the gel and Alpha EaseFC software densitometry tool was used to obtain a relative quantification by calculating the sum of the pixels in the area of the band sum and subtracting the background. Each band was normalized by dividing by the amount of *GAPDH* control.

Immunohistochemistry

Anti-MMP-11 clone SL3.05 from Labvision/Neomarkers (Fremont, CA) was used at a 1:30 dilution. The slides were deparaffinized using a series of xylene washes and graded ethanol washes, as described (27). Heat Induced Epitope Retrieval was performed using a Biocare Decloaker (Concord, CA) with 1X Citra-Plus Buffer from Biogenex (San Ramon, CA). All slides were treated with 3% hydrogen peroxide (Fisher Scientific, Fairlawn, NJ) for 5 min., followed by 10% Normal Goat Serum (Vector Laboratories, Burlingame, CA) incubation for 20 min in a humidity chamber. Primary antibodies were applied for 1 hr in a humidity chamber. Detection was performed using Envision (Dakocytomation, Carpinteria, CA) for 30 min in a humidity chamber. Diaminobenzidine (DAB+) solution (Dakocytomation) was applied for 10 min., followed by a 2 min. hematoxylin counterstain. Slides were rinsed between each step with phospho-buffered saline with Tween. Slides were then dehydrated with a series of graded ethanol washes and xylene washes, mounted with Permount (Fisher Scientific, Pittsburgh, PA) and cover-slipped for bright field microscopy.

Western blotting

Frozen tumor samples were pulverized into powder and lysed in buffer (50 mmol/L Tris pH 7.4, 0.15 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholate) supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany). Samples were vortexed for one minute, then homogenized using a Polytron (Brinkmann Instruments, Westbury, NY). Tumor lysates were cleared by centrifugation at 13,000 g for 10 minutes, and protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Aliquots were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane and processed as described (28). MST-4 and PKC-β1 were assessed using an anti-MST-4 antibody (BD, Franklin Lakes, NJ) and a polyclonal anti-PKC-β1 antibody (C-16 Santa Cruz Biotechnology, Santa Cruz, CA) respectively. Equal loading was assessed using a monoclonal anti-GAPDH antibody (IMGEX, San Diego, CA). Band intensity was quantitated by densitometry using Quantity One software (V4.5.1) and a GelDoc Imaging System (Bio-Rad).

Microarray Analysis

Initial quality assessment of all scanned chips was performed using GeneChip Operating Software (GCOS) v1.1 (Affymetrix, <http://www.affymetrix.com>). Compiled data in the form of six individual CEL files, the primary output of scanned Human Genome U133 plus 2.0 microarray chips, were imported to GeneSpring (Agilent Technologies, <http://www.chem.agilent.com>) for analysis using the native probe level GC-Robust Multi-array Average (GC-RMA) algorithm (29).

GC-RMA calculation performs an initial background noise/non-specific binding correction using the G-C content to estimate hybridization affinities of the individual targets that comprise a set of probes for any given gene. Next, the GC-RMA algorithm consists of three further steps. In the first step, the affinity corrected mis-match (MM) hybridization scores are subtracted from perfect match (PM) hybridization scores for every target of a given probe set. Secondly, a quantile non-linear normalization is performed that corrects for bias among arrays in the full data set, such that each array can be directly compared to any other. Finally the quantile normalized probe level data, derived from the initial background noise/non-specific binding correction step, are summarized as a single expression measurement for that given probe set. When compared to previous data processed using MAS v5.0 methods, GC-RMA showed better precision evidenced by lower variance within replicate samples especially at low levels of gene expression. Further, GC-RMA seems to have more consistent estimates of fold change among arrays as well as a lower rate of false discovery (30).

Following GC-RMA analysis of the six batched CEL files, the data were grouped according to normal or diseased parameters and treated as biologic triplicate samples. Initially, the data were filtered for genes that scored above 20 RAW intensity units on at least three of the six arrays, so that a gene could be completely unreliable (absent) in one condition but have reliable expression in the other (27,822 genes pass). Because the Log Ratio of normalized data is centered on 1.0, genes that were very similar to 1.0 in both conditions measured by a standard t-test with a multiple testing correction False Discovery Rate (FDR) from 0.95 to 1.00 were removed yielding 25,331 genes. In the next filter, genes that were equal in expression by up to 10% of the mean value were removed leaving 16,459 genes. The last filter that was applied to the data set identified potentially differential genes from the preceding list with values that were greater or less than 1.25 fold in each condition (7,470 genes pass).

The final output list from the filtering process was used as input for ANOVA statistical testing. In order to find a list of statistically significant genes with high degree of confidence, a multiple testing correction FDR of 0.10 (10%) was chosen, and the analysis restricted to genes showing a minimum of two fold change. We identified a total of 816 significant genes that show differential expression between these two conditions (with the caveat that 10% [~82 genes] of these genes may not differ statistically). The genes resultant from the statistical test were then grouped by direction of expression change (up or down between the two conditions) and filtered for a greater than 2.0 fold change. We found 97 genes that showed a significant increase and 619 genes with a significant decrease in the leiomyomata as compared to the matching normal tissue.

RESULTS

We decided to study multiple tumors from a single patient to eliminate inter-subject genetic variability and differences in hormonal milieu. Our patient (B5) underwent a hysterectomy for symptomatic uterine fibroids (namely pain). She was 53 years old and was established to be postmenopausal based on histological examination of her endometrium that was found to be atrophic. She had not received any treatment for her fibroids prior to her hysterectomy. We obtained tissue from three tumors ranging in size between 4 and 11 cm, and adjacent

myometrium and selected matched pairs for Affymetrix microarray analysis as described in Materials and Methods.

As shown in figure 1A, of the 38,500 genes contained in the array, we identified 816 that exhibited significant differences according to our criteria. We focused on genes that showed consistent changes in expression between normal and diseased state, in the same direction, in all 3 paired samples. Statistically significant changes were further selected by including only those genes changing by a magnitude of two-fold or greater. Of the 816 differentially expressed genes that met our criteria, 619 were down-regulated and 97 were up-regulated by two fold. Functional categories of genes showing altered expression included: regulators and components of the extracellular matrix, metabolism, signal transduction, apoptosis, transcription factors, protein trafficking and cell cycle regulation. This preponderance of down regulated genes in uterine leiomyomata is consistent with previous analyses. Cluster analysis of the independent samples (Fig. 1B) demonstrates some variability in the absolute expression levels between tumors and between normal tissues. However the overall profiles of the 3 fibroids and the 3 myometrial samples respectively were similar. Thus, individual tumors derived from a single patient, with identical genetic factors and hormonal milieu, showed consistent changes in mRNA relative to normal myometrium.

Selected genes were chosen for confirmation using a panel of paired normal myometrium and fibroid samples, including the samples (B5) used for the array, but also matched myometrium and leiomyoma tissue obtained from multiple additional patients. This procedure allowed us to not only confirm the changes observed in the array analysis but also to assess the inter-patient and for a given patient, inter-tumor variability of expression. Confirmatory methods utilized were semi-quantitative RT-PCR, Western blot and immunohistochemistry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for protein or mRNA levels, respectively. Levels were similar in leiomyomata and myometrial samples. In contrast, actin and β -tubulin exhibited considerable variation in expression in leiomyoma and were not suitable controls (not shown). Fibulin 3 (EGF-containing fibulin-like extracellular matrix protein 1) has been identified by arrayanalysis from other laboratories as being differentially expressed (13,18,23,24); it was down-regulated approximately 20-fold in our analysis. In subsequent RT-PCR analysis, fibulin 3 was shown to be down regulated in the majority of tumors examined, with the exception of one of five tumors from patient B9 (Fig. 2A). Similarly, downregulation of the matrix metalloproteinase MMP7 (4.37-fold) was confirmed by RT-PCR in twelve out of thirteen tumors derived from five additional patients (Fig 2C). This gene had not been identified as differentially expressed prior to our analysis.

We also identified desmoglein 2, a member of a subclass of desmosomal cadherins, as a novel target not previously detected by array analyses. Desmoglein 2 was significantly up-regulated (5-fold) in leiomyomata. This finding was confirmed by RT-PCR in additional patients and tumors (Fig. 2B). Some inter and intra-patient variability was also observed with two tumors exhibiting decreased desmoglein 2 levels relative to adjacent myometrium.

In contrast to MMP7, stromelysin 3, or MMP11, was found to be significantly up-regulated in leiomyomata, consistent with previous reports (10,11,19,24), (Fig. 3A). The 5–10 fold increased expression of MMP11 by array and RT-PCR was confirmed by immunohistochemical analysis of several tumors. As shown in figure 3B, leiomyomata derived from multiple patients stained significantly more intensely for MMP11, relative to matched normal myometrium. No staining was detected using non-immune immunoglobulin (not shown).

Finally, we examined expression of MST4 (Mst3 and SOK1-related kinase), a member of a family of serine/threonine kinases that may regulate apoptotic pathways, and protein kinase C

$\beta 1$ (PKC $\beta 1$). Array analysis indicated that PKC $\beta 1$ was up-regulated approximately 5-fold in leiomyoma while levels of MST-4 exhibited a significant (>20-fold) decrease in tumors. These results were confirmed by western blot analysis of tissue extracts from multiple additional patients (Fig. 4). Blots were scanned and quantitated, and levels normalized to GAPDH. Upregulation of PKC $\beta 1$ was observed in 12 of 15 matched tumor and normal samples (Fig. 4A), while MST4 was down-regulated in the majority (10 of 15) leiomyomata (Fig. 4B). Downregulation of MST4 was also observed in the majority of tumors (12 of 13) analyzed by RT-PCR (Fig. 2D).

Automated global analysis of the genes that were significantly altered in expression in leiomyomata using GeneSpring Gene Ontology (GO) and Ingenuity Pathway Analysis (<http://www.ingenuity.com/>) did not identify any obvious signaling pathways or functional categories when queried with the statistically significant gene lists. Thus, to provide additional insight into the pathophysiology of uterine leiomyomata we reviewed the literature, focusing on genes that have been described previously as relevant in malignant disease. NCBI databases were searched using the gene symbol or other gene designations and available information about a particular gene reviewed using GeneRIF (Gene Reference Into Function <http://www.ncbi.nlm.nih.gov/projects/GeneRIF/>). This analysis identified changes in the expression of genes linked to cell growth, survival, apoptosis, invasion and metastasis which are shown in Table 1. We also identified putative oncogenes and genes typically up-regulated in cancers. Overall, leiomyomata exhibited increased expression of genes associated with cell proliferation and survival (e.g. IGF-2 TGF $\beta 3$, PKC $\beta 1$, cyclin D and bcl-2) and a decrease in levels of pro-apoptotic genes (e.g. MST4 and TRAIL) relative to normal myometrium. Interestingly, whilst a number of putative tumor suppressors were down-regulated in leiomyoma, genes typically up-regulated in cancers were found to be down-regulated in these benign uterine tumors. This observation was particularly evident with respect to genes typically over expressed in invasive or metastatic cancers. Such genes were generally expressed in leiomyomata at lower levels than normal myometrium (Table 1), with the exception of the matrix metalloproteinases 11 and 14.

DISCUSSION

To date over 15 microarray analyses of uterine fibroids have been published. However, there is considerable variation and lack of overlap between studies, even those utilizing similar platforms and methodology. Consequently, alterations in gene expression that are functionally relevant to the pathophysiology of uterine leiomyoma are not clearly understood (26). These apparent disparities in gene profiles cannot be fully explained by methodological differences between the studies and may be attributable in part to genetic variability between patients, differences in the hormonal milieu or distinct chromosomal alterations between tumors (31). These factors may contribute to intrinsic variability of gene expression in the normal myometrium and/or fibroids. Last but not least, there is the possibility of existence of distinct fibroid subtypes (32–37), analogous to other diseases like leukemia, breast and lung cancer (38). In order to circumvent and control for the above possible sources of variation, we elected to study multiple leiomyomata samples and matched normal myometrium obtained from a single patient, i.e. in the context of an identical hormonal and genetic environment. Changes in expression of selected genes were then confirmed by analysis of multiple paired samples derived from additional patients.

In our study we examined the expression of nearly 38,500 gene fragments in independent RNA isolates from 3 fibroids and 3 normal samples from the same patient using Affymetrix technology. Statistically significant changes in gene expression were further limited to those probe sets exhibiting consistent changes in all three tumors of at least two-fold in magnitude, compared to average expression in normal myometrium. Using this approach, we found 816

genes to be differentially expressed in leiomyomata compared to normal myometrium. Consistent with previous studies, the majority of genes (619) were down-regulated in fibroids. We identified a number of genes in common with previous fibroid microarrays including some with otherwise disparate results (10–25). Thus our gene profile exhibited overlap with several apparently discordant studies, perhaps indicative of the sub set of common physiological relevant changes in expression in these tumors. Interestingly the least amount of overlap was noted with results using RNA derived from primary cell cultures of myometrium and leiomyomata (14). Differences in gene expression between primary and immortalized cell lines compared to their corresponding tumors and normal tissue have been previously described (39) and may be a reflection of removal of the cells from their endogenous environment, hormonal milieu and extracellular matrix.

We confirmed differential expression of several genes, namely fibulin 3, desmoglein 2, MMP7, MMP11, MST4 and PKC β 1 using three independent methods including RT-PCR, immunohistochemistry and Western blotting, to show changes not only at the level of the message, but also in some cases at the level of protein.

Among genes altered in expression were several fibulins, members of a family of secreted glycoproteins, characterized by repeated epidermal-growth-factor-like domains and a unique C-terminal structure. Evidence indicates a structural role for fibulins within the extracellular matrix and they have also been shown to modulate cell morphology, growth, adhesion and motility. The dysregulation of certain fibulins occurs in a range of human disorders, including cancer and both tumor suppressive and oncogenic activities have been proposed for members of the fibulin family (40).

Consistent with previous reports (14,20,41,42), fibulin 1 was over expressed (2.2 fold) in leiomyoma. Fibulin 1 is a TGF β regulated gene (42), which inhibits cancer cell adhesion, motility and invasion in vitro (43–45) and tumor growth in vivo (45). In contrast, fibulin 2 is a novel target identified in our study as down-regulated (2.5 fold) in leiomyoma. The role of fibulin 2 in fibroids and other neoplasms is not clear.

Fibulin 3 or also called EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) was also down-regulated (20-fold) in our array and utilizing RT-PCR we confirmed decreased expression levels in our study patient and 5 additional patients (Fig. 2). Previous studies also identified this gene as down-regulated in leiomyoma (13,18,23,24). The related protein, fibulin 5 also exhibited 2-fold lower expression in leiomyoma. Fibulin 3 and 5 have been shown to inhibit angiogenesis in tumors and may function as tumor suppressors (46–48). However, fibulin-3 expression is increased in a range of transformed cell lines compared with normal controls (49) and the functional role of fibulins 3 and 5 in leiomyoma has not been established. Interestingly fibulin 5 knockout mice showed enhanced proliferation of vascular smooth muscle in response to mitogens (50) consistent with a possible inhibitory role for fibulin 5 in regulating myometrial cell proliferation.

Desmoglein 2 (DSG2) is a novel up-regulated (5-fold) target not identified previously. Over expression of DSG2 is associated with increased growth rate, resistance to apoptosis and increased cell survival (51,52). Desmoglein 2 is a component of adherence junctions and has been implicated in invasion and metastasis of squamous cell carcinomas. Decreased DSG2 expression is linked to loss of differentiation and poorer prognosis in gastric cancers (53).

MST 4 is a serine/threonine protein kinase and inducer of apoptosis and its over expression has been implicated in prostate cancer metastasis (54). In agreement with a previous study (11), we found MST4 to be dramatically down-regulated (20–30 fold) in leiomyoma. Decreased expression of MST4 was confirmed in multiple patients by RT-PCR and Western blot analysis (Figs. 2 & 4B).

PKC β 1 has been identified as up-regulated in leiomyoma by one other group (11). We found a 4.9 fold increase in PKC β 1 expression and confirmed increased protein levels in multiple patients (Fig. 4A). The role of specific PKC isoforms in leiomyoma has not been extensively investigated. PKCs have been implicated in endothelin 1 growth stimulation of uterine leiomyoma (55) and conversely, growth inhibition of leiomyoma, by the GnRH analog buserelin, is also PKC-dependent (56).

A number of MMPs exhibit increased expression in leiomyoma, including MMP-11 (stromelysin 3) (10,11,19,24,57) and MMP 14 consistent with aberrant extracellular matrix deposition. In contrast, MMP-7 (matrilysin) is a target identified for the first time by our microarray as down-regulated (4.9 fold) in leiomyoma relative to myometrium. MMP-7 is a matrix-metalloproteinase that influences tumor progression by regulating invasion and angiogenesis. MMP-7 status of cancer tissues has been shown to be a strong predictor of poor prognosis (58,59). Serum MMP-7 levels are significantly elevated in patients with advanced colorectal cancer and are an independent prognostic factor for survival (60). MMP-7 is over-expressed in malignant ovarian epithelium and may facilitate tumor cell invasion in vivo (61). Increased expression of MMP-7 in high grade uterine endometrial carcinoma is also associated with tumor invasion and metastasis (62).

In our gene screen and confirmatory experiments we noticed some variation in expression both on the message and protein level. Variation in protein levels was typically less than transcript levels. However, the pattern of expression was generally consistent between transcript and protein levels (Figs 2 & 4), and discordant profiles, with respect to the array analysis, were observed in both contexts. Expression levels of our selected targets varied between tumors from the same patient and also among tumors from different patients. Overall, variation in expression between tumors in a given patient was less than inter-patient variability. However, we also observed considerable differences, in the magnitude and direction of change, between tumors in a single patient (Fig 2–4).

Genetic variability in fibroids has been described in detail in the past, ascribed to different chromosome alterations and certain genetic mutations (31,63,64). These observations suggest that several fibroid subtypes exist similarly to lymphomas, breast and lung cancer, characterized by differential gene expression profiles.

With the exception of components of retinoid metabolism (65–68) and TGF- β signaling, automated pathway or gene ontology analysis of the leiomyoma gene profile, using GeneSpring and Ingenuity software respectively, failed to show coordinated changes in specific pathways. For this reason, we reviewed functional annotations for each gene using the GeneRIF (Gene Reference Into Function) database (National Library of Medicine). This analysis revealed several functional groups relating to regulation of apoptosis, cell growth and invasion and metastasis (Table 1). It is evident that, in general, genes linked to the promotion of growth, and inhibition of apoptosis are up-regulated in leiomyoma, whilst proapoptotic and growth inhibitory genes are down-regulated. This pattern is consistent with enhanced proliferation and survival of leiomyoma relative to normal myometrium. We also observed that genes typically associated with malignant disease and up-regulated in invasive and metastatic tumors, were actually down-regulated in leiomyoma relative to normal myometrium. This suppression of invasion and metastasis related gene expression may underlie the benign, highly-differentiated, non-invasive phenotype characteristic of leiomyoma. Moreover, we speculate that this functional gene group may provide a molecular signature distinguishing leiomyoma from its malignant counterpart leiomyosarcoma, facilitating differential diagnosis and treatment at an early stage. Consistent with this hypothesis, previous studies have identified several genes up-regulated in leiomyosarcomas compared to normal myometrium that were actually down-regulated in our study and belong to our invasion, migration and metastasis

group, e.g. tenascin C and WNT1 inducible signaling pathway protein 1 (69,70). Early leiomyosarcoma and leiomyoma can exhibit similar symptomatology and imaging characteristics making preoperative diagnosis difficult. Histological sections can also be ambiguous, thus leading to critical delays in appropriate referral and therapeutic intervention (71). In future work, we will evaluate the relative expression levels and prognostic value of this invasion and metastasis gene set in leiomyoma and leiomyosarcoma.

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A

B

Figure 1.

A. The statistically significant differentially expressed genes that exhibited consistent changes in all 3 tumors relative to normal myometrium of a magnitude of at least 2 fold. B. Heat map showing cluster analysis of the differentially expressed 816 genes in 3 uterine leiomyoma and 3 samples of matched adjacent normal myometrium. From left to right the first 3 columns represent the 3 tumors followed by the corresponding normal tissue. Rows represent individual genes. Red, increased gene expression; green, decreased gene expression. The color intensity is proportional to the hybridization intensity of a gene from its median level across all samples.

Figure 2.

Semiquantitative RT-PCR analysis. RNA from paired myometrium/leiomyoma from 5 different patients was analyzed using specific primer pairs, as indicated and described in Materials and Methods and electrophoresed on agarose gels. Bands were quantitated by scanning densitometry and expressed as relative intensity normalized to GAPDH. B2, B3, B4, B9, B11 – patient designations. N1-5 normal myometrium; L1-5 corresponding leiomyoma samples.

Figure 3.

A. Semiquantitative RT-PCR analysis of MMP11 (Stromelysin 3) expression. RNA from paired myometrium/leiomyoma from 5 different patients was analyzed using specific primer pairs, as indicated and described in Materials and Methods and electrophoresed on agarose gels. Bands were quantitated by scanning densitometry and expressed as relative intensity normalized to GAPDH. B2, B3, B4, B9, B11 – patient designations. N1-5 normal myometrium; L1-5 corresponding leiomyoma samples.

B. Immunohistochemical staining for matrix metalloproteinase 11 (MMP-11) in normal myometrial tissue (top panels) and leiomyomata (bottom panels). Paraffin embedded sections were stained with anti-MMP-11 antibody as described in Materials and Methods.

Representative 20X fields are depicted. B2, B3, B4, B9, B11 patient designations. N: normal myometrium, L matched leiomyoma.

Figure 4.

Western blots analysis of leiomyoma and myometrium samples from 5 patients using antibodies to A. PKC β I and B. MST-4. Blots were reprobed for GAPDH as a loading control. Chemiluminescence was quantitated using a BioRad Chemdoc XRS imager and relative levels expressed as a ratio of leiomyoma to normal myometrium, normalized to GAPDH. B2, B4, B5, B8 and B9; patient designations, numbers indicate independent tumor/normal paired samples derived from that patient. N: myometrium, L: leiomyoma.

Table 1

Differentially expressed genes in leiomyomata categorized by the indicated functional groups based on published reports. Multiple entries reflect different probe sets for a given target. Genes are listed in order of magnitude of fold change relative to normal myometrium. Negative values indicate down regulated genes.

Proapoptotic genes			
Genbank	Description	Fold	Gene Symbol
AF344882	Mst3 and SOK1-related kinase	-31.55	MST4
NM_016542	Mst3 and SOK1-related kinase	-22.72	MST4
NM_002583	PRKC, apoptosis, WT1, regulator	-2.84	PAWR
NM_004938	Death-associated protein kinase 1	-2.34	DAPK1
AF356193	Caspase recruitment domain family 6	-2.28	CARD6
BF434846	Tenascin C (hexabrachion)	-4.80	TNC
NM_006307	Sushi-repeat-containing protein, X-linked	-2.04	SRPX
AI281371	APO-1/CD95 (Fas)-associated phosphatase	-3.98	PTPN13
NM_006264	APO-1/CD95 (Fas)-associated phosphatase	-2.39	PTPN13
AW770896	Insulin-like growth factor binding protein 7	-2.30	IGFBP7
NM_002184	IL6 signal transducer (oncostatin M receptor)	-4.71	IL6ST
AW242916	IL6 signal transducer (oncostatin M receptor)	-2.05	IL6ST
AB015706	IL6 signal transducer (oncostatin M receptor)	-2.05	IL6ST
NM_002135	Nuclear receptor subfamily 4A1	-2.02	NR4A1
BC002439	HIV-1 Tat interactive protein 2, 30kDa	-2.86	HTATIP2
BF511276	A kinase anchor protein (gravin) 12	-2.75	AKAP12
BF511276	A kinase anchor protein (gravin) 12	-2.42	AKAP12
AB003476	A kinase anchor protein (gravin) 12	-2.19	AKAP12
U57059	TNF-related apoptosis inducing ligand TRAIL	-2.17	TNFSF10
NM_004226	Ser/thr kinase 17b (apoptosis-inducing)	-3.96	STK17B
AA203487	Ser/thr kinase 17b (apoptosis-inducing)	-2.26	STK17B
NM_021730	CARD7, DEFCAP	2.18	NALP1
AF003934	Growth differentiation factor 15	5.23	GDF15
Growth promoting genes			
Genbank	Description	Fold	Gene Symbol
NM_003294	Tryptase alpha/beta 1	2.02	TPSAB1
NM_001897	Chondroitin sulfate proteoglycan 4	2.32	CSPG4
NM_002997	Syndecan 1	2.26	SDC1
AV694854	Adrenergic, alpha-1A-, receptor	3.01	ADRA1A
N51516	Adrenergic, alpha-1A-, receptor	2.53	ADRA1A
AJ000008	Phosphoinositide-3-kinase, gamma	2.87	PIK3C2G
BC003105	Protein tyrosine phosphatase type IVA, 3	2.05	PTP4A3
X99268	Twist homolog 1	2.17	TWIST1
NM_000612	Insulin-like growth factor 2 (somatomedin A)	5.67	IGF2
M17863	Insulin-like growth factor 2 (somatomedin A)	3.55	IGF2

Proapoptotic genes

Genbank	Description	Fold	Gene Symbol
J03241	Transforming growth factor, beta 3	2.37	TGFB3
NM_002738	Protein kinase C, beta 1	4.90	PRKCB1
M13975	Protein kinase C, beta 1	2.98	PRKCB1
M73554	Cyclin D1 (PRAD1)	2.74	CCND1
NM_001759	Cyclin D2	2.06	CCND2
NM_005195	CCAAT/enhancer binding protein delta	-2.07	CEBPD
NM_002039	GRB2-associated binding protein 1	-2.24	GAB1

Growth inhibitory genes

Genbank	Description	Fold	Gene Symbol
AI281593	Decorin	-2.45	DCN
AF138303	Decorin	-2.09	DCN
AI343467	Inhibin, beta A (activin A, activin AB alpha)	-3.99	INHBA
M13436	Inhibin, beta A (activin A, activin AB alpha)	-3.17	INHBA
NM_002036	Duffy blood group	-2.95	DARC
AA530892	Dual specificity phosphatase 1 (MKP-1)	-2.63	DUSP1
AW770896	Insulin-like growth factor binding protein 7	-2.30	IGFBP7
AF493929	Regulator of G-protein signalling 5	-2.18	RGS5
AI183997	Regulator of G-protein signalling 5	-2.07	RGS5
AF132818	Kruppel-like factor 5	-5.05	KLF5
AF003114	Cysteine-rich, angiogenic inducer, 61	-8.12	CYR61
NM_001554	Cysteine-rich, angiogenic inducer, 61	-6.59	CYR61
AA150501	Epithelial membrane protein 1	-2.32	EMP1
BE552421	Mitochondrial tumor suppressor 1	-2.22	MTUS1
AW444761	Cyclin-dependent kinase inhibitor 2B (p15)	-3.41	CDKN2B
NM_022470	P53 target zinc finger protein	2.29	ZMAT3

Putative Tumor Suppressor genes

Genbank	Description	Fold	Gene Symbol
L16895	Lysyl oxidase	-2.61	LOX
NM_002317	Lysyl oxidase	-2.31	LOX
AF101051	Claudin 1	-13.02	CLDN1
NM_021101	Claudin 1	-5.33	CLDN1
NM_006307	Sushi-repeat-containing protein, X-linked	-2.04	SRPX
M92934	Connective tissue growth factor	-3.34	CTGF
NM_006207	Platelet-derived growth factor receptor-like	-2.67	PDGFRL
NM_012307	Erythrocyte membrane protein band 4.1-like 3	-2.00	EPB41L3
BE552421	Mitochondrial tumor suppressor 1	-2.22	MTUS1

Proapoptotic genes

Genbank	Description	Fold	Gene Symbol
NM_003206	Transcription factor 21	-11.05	TCF21
AF055585	Slit homolog 2	-3.00	SLIT2
AI692523	Slit homolog 2	-2.95	SLIT2
AI963304	Slit homolog 2	-2.36	SLIT2
AI343467	Inhibin, beta A (activin A, activin AB alpha)	-3.99	INHBA
M13436	Inhibin, beta A (activin A, activin AB alpha)	-3.17	INHBA
AF017987	Secreted frizzled-related protein 1	-2.28	SFRP1
AI332407	Secreted frizzled-related protein 1	-2.07	SFRP1
NM_003012	Secreted frizzled-related protein 1	-2.02	SFRP1
U16153	Inhibitor of DNA binding 4	-2.58	ID4
AW157094	Inhibitor of DNA binding 4	-3.47	ID4
NM_013253	Dickkopf homolog 3	-2.26	DKK3
AU148057	Dickkopf homolog 3	-2.23	DKK3
NM_002318	Lysyl oxidase-like 2	2.74	LOXL2
U17074	Cyclin-dependent kinase inhibitor 2C (p18)	2.63	CDKN2C
NM_001262	Cyclin-dependent kinase inhibitor 2C (p18)	2.63	CDKN2C

Genes associated with invasion, migration or metastasis

Genbank	Description	Fold	Gene Symbol
AF101051	Claudin 1	-13.02	CLDN1
NM_021101	Claudin 1	-5.33	CLDN1
NM_004938	Death-associated protein kinase 1	-2.34	DAPK1
NM_006614	Cell adhesion molecule with homology to L1CAM	-24.44	CHL1
BF434846	Tenascin C (hexabrachion)	-4.80	TNC
AL552534	CD44 antigen	-2.71	CD44
AF098641	CD44 antigen	-2.40	CD44
M24915	CD44 antigen	-2.38	CD44
NM_000610	CD44 antigen	-2.32	CD44
AI493245	CD44 antigen	-2.13	CD44
BC004372	CD44 antigen	-2.12	CD44
J05021	Villin 2 (ezrin)	-2.22	VIL2
AA670344	Villin 2 (ezrin)	-2.19	VIL2
AL574210	Serine proteinase inhibitor, PAI 1	-3.73	SERPINE1
U66495	Leptin receptor	-2.48	LEPR
U08626	Leptin receptor	-2.46	LEPR
AI308863	Leptin receptor	-2.05	LEPR
NM_000956	Prostaglandin E receptor 2 (subtype EP2)	-2.25	PTGER2
NM_003882	WNT1 inducible signaling pathway protein 1	-2.48	WISP1
AA147884	WNT1 inducible signaling pathway protein 1	-2.07	WISP1

Proapoptotic genes

Genbank	Description	Fold	Gene Symbol
AI917494	WNT1 inducible signaling pathway protein 1	-2.07	WISP1
NM_007003	P antigen family, member 4	-3.07	PAGE4
NM_001993	Coagulation factor III (tissue factor)	-4.21	F3
NM_002010	Fibroblast growth factor 9	-5.19	FGF9
AI520969	Vimentin	-2.08	VIM
AF344882	Mst3 and SOK1-related kinase	-31.55	MST4
NM_016542	Mst3 and SOK1-related kinase	-22.72	MST4
AA749101	Interferon induced transmembrane protein 1	-2.14	IFITM1
NM_002276	Keratin 19	-11.30	KRT19
NM_002423	Matrix metalloproteinase 7 (matrilysin)	-4.37	MMP7
AI417595	Endomucin	-2.94	EMCN
AI635774	Endomucin	-2.07	EMCN
AI281371	APO-1/CD95 (Fas)-associated phosphatase	-3.98	PTPN13
NM_006264	APO-1/CD95 (Fas)-associated phosphatase	-2.39	PTPN13
AI189753	Human tumor antigen (L6), TAAL6	-2.26	TM4SF1
M90657	Human tumor antigen (L6), TAAL6	-2.71	TM4SF1
U76833	Fibroblast activation protein, alpha	-2.30	FAP
L01639	Chemokine (C-X-C motif) receptor 4	-2.21	CXCR4
AF348491	Chemokine (C-X-C motif) receptor 4	-2.06	CXCR4
D13889	Inhibitor of DNA binding 1, Id1	-2.98	ID1
AF003114	Cysteine-rich, angiogenic inducer, 61	-8.12	CYR61
NM_001554	Cysteine-rich, angiogenic inducer, 61	-6.59	CYR61
AL136139	Enhancer of Filamentation 1	-2.50	HEF1
NM_004995	Matrix metalloproteinase 14	2.65	MMP14
Z48481	Matrix metalloproteinase 14	2.21	MMP14
X83535	Matrix metalloproteinase 14	2.06	MMP14
NM_005940	Matrix metalloproteinase 11 (stromelysin 3)	7.58	MMP11
AI761713	Matrix metalloproteinase 11 (stromelysin 3)	3.80	MMP11
X99268	Twist homolog 1	2.17	TWIST1