Experimental and Molecular Pathology 110 (2019) 104284

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Identification of a novel diagnostic gene expression signature to discriminate uterine leiomyoma from leiomyosarcoma

Crystal L. Adams[a], Irina Dimitrova[a,b], Miriam D. Post[c], Lacey Gibson[a], Monique A. Spillman[a,b], Kian Behbakht[b], Andrew P. Bradford[a,*]

[a] Department of Obstetrics and Gynecology, Division of Reproductive Sciences, University of Colorado, Anschutz Medical Campus, Aurora, CO, USA
[b] Department of Obstetrics and Gynecology, Division Gynecologic Oncology, University of Colorado, Anschutz Medical Campus, Aurora, CO, USA
[c] Department of Pathology, University of Colorado, Anschutz Medical Campus, Aurora, CO, USA

ARTICLE INFO

Keywords:
Leiomyosarcoma
Leiomyoma
Gene expression
Molecular profiling
Diagnosis

ABSTRACT

Leiomyosarcomas are rare, aggressive tumors, which exhibit a poor prognosis regardless of stage. Pre-operative diagnosis can be difficult as leiomyosarcoma can mimic features of the more common, benign uterine leiomyoma. The goal of this study was to identify specific molecular markers to discriminate between uterine leiomyosarcomas and leiomyomas to facilitate timely, accurate diagnosis and treatment.

Gene expression profiles of three leiomyosarcomas, leiomyomas, and normal myometrial tissue samples were analyzed using the Affymetrix Human Gene 1.0 ST Array. GC-robust multiarray average calculation and ANOVA statistical testing were used to identify differentially expressed genes. Sixty genes, with functional roles in tumor progression or suppression, exhibited divergent expression profiles in leiomyosarcomas and leiomyomas, compared to normal myometrium. Differential RNA and protein levels of seven genes, with the most discriminatory expression patterns, were confirmed by RTPCR and immunohistochemistry in an additional 10 leiomyosarcoma and 20 leiomyoma independent samples. *CHI3L1*, *MELK, PRC1, TOP2A,* and *TPX2* were overexpressed in leiomyosarcomas, while *HPGD* and *TES* were overexpressed in leiomyomas. Distinguishing leiomyosarcomas from leiomyomas represents a diagnostic challenge, particularly in the context of minimally invasive surgery. The unique gene expression signatures identified in this study may accurately differentiate between these tumor types at the earliest stage and provides potential prognostic factors and novel therapeutic targets for the treatment of leiomyosarcoma.

1. Introduction

Uterine leiomyosarcoma (LMS) is a rare malignancy, comprising approximately 1% of all uterine cancers and one-third of uterine sarcomas (Echt et al., 1990). Incidence estimates range from 0.61–1.5 per 100,000 women annually (Brooks et al., 2004). While reported cases of metastatic uterine LMS date back to the early 1900s, methods of pre-operative diagnosis and treatment remain limited. Despite the majority presenting with stage I disease, prognosis for uterine LMS is uniformly poor, with an overall 5-year disease-specific survival rate of just 66% (Kapp et al., 2008).

In contrast to LMS, uterine leiomyomas (LMA) are common, benign myometrial neoplasms with an estimated cumulative incidence of approximately 70%–80% in US women by age 50 (Baird et al., 2003). Uterine LMA are not thought to develop into LMS. However, depending on size and location, LMA may cause abnormal uterine bleeding, pelvic pain, dysmenorrhea, and dyspareunia.

Clinically, uterine LMS and LMA are often difficult to distinguish. In the absence of disseminated disease, they present with identical exam findings. Ultrasonographic markers such as heterogenous echogenicity, abnormal vascular distribution, and central necrosis, may be present in either tumor type (Amant et al., 2009). Furthermore, with an endometrial sampling sensitivity of 38–67% for uterine LMS (Bansal et al., 2008), no conclusive preoperative biopsy methods currently exist (Mahnert et al., 2015). This represents a significant diagnostic and therapeutic dilemma due to similar clinical presentations, but markedly different treatment and outcomes.

Over 200,000 hysterectomies are performed annually for uterine fibroids. The rate of laparoscopic hysterectomy has also increased steadily (Farquhar and Steiner, 2002). Minimally invasive

* Corresponding author at: Department of Obstetrics & Gynecology, Division of Reproductive Sciences, University of Colorado, Anschutz Medical Campus, 12700 E. 19th Ave, RC-2, Mail Stop 8613, Aurora, CO 80045, USA.
E-mail address: Andy.Bradford@ucdenver.edu (A.P. Bradford).

https://doi.org/10.1016/j.yexmp.2019.104284
Received 2 April 2019; Received in revised form 3 June 2019; Accepted 9 July 2019
Available online 10 July 2019

hysterectomy and myomectomy have many benefits, including fewer postoperative complications, less blood loss, reduced pain, and faster recovery. However, these procedures may require the use of morcellation to complete tissue extraction. In 2014, the FDA estimated that the prevalence of unsuspected LMS was 1 in 498 for patients undergoing surgery for presumed benign LMA Center for Devices and Radiological Health, 2014, with similar estimates ranging from 1 in 400 to 1 in 1000 (Worldwide, 2014). Inadvertent morcellation of occult uterine LMS is associated with higher recurrence and death rates (Bogani, 2015; Ricci et al., 2017). Thus, preoperative differentiation between these tumor types is of paramount importance to clinical management and prognosis.

Because intraoperative frozen section is not reliable for excluding uterine LMS, definitive postoperative diagnosis is imperative. The Stanford criteria utilize mitotic index, cytologic atypia and coagulative necrosis to diagnose uterine smooth muscle tumors. Histologic evaluation and diagnosis of LMS may be challenging, due to varying degrees of one or more criteria (Kempson and Hendrickson, 2000). Moreover, benign LMA may exhibit increased mitotic activity, marked cytologic atypia and areas of hyalinizing necrosis (Lee et al., 2009). Thus, molecular techniques to differentiate these tumor types may improve pre- and postoperative diagnostic accuracy.

Prior studies have evaluated gene expression differences among LMS of multiple primary origins (Baird et al., 2005; Lee et al., 2004; Miyajima et al., 2001; Quade et al., 2004; Rao et al., 1999; Skubitz and Skubitz, 2003). However, comparisons are difficult, given differing gene array platforms, statistical analyses, study designs and limited validation at the protein level. Aims range from broad differentiation of LMS and sarcomas (Baird et al., 2005), to identification of isolated, predetermined gene expression profiles (Miyajima et al., 2001), to evaluation of prognostic grouping criteria (Lee et al., 2004). Few studies specifically evaluate gene expression differences between uterine LMS and LMA or normal myometrium (Quade et al., 2004). While LMS of uterine and extra-uterine origin exhibit a considerable level of homogeneity (Baird et al., 2005; Skubitz and Skubitz, 2003), significant prognostic differences in gene expression, mutation, and amplification have been demonstrated (Rao et al., 1999).

In this study, we utilized cDNA microarray to identify differences in gene expression among uterine LMS and LMA, compared to normal myometrium. For purposes of validation, we selected seven genes, with the greatest differential expression and a previously determined role in gynecologic cancers, for further evaluation by semiquantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC). Our study is the first to identify a unique gene expression signature to potentially distinguish uterine LMS from benign LMA with clinical implications for diagnosis, prognosis, and therapeutic modalities.

2. Materials and methods

2.1. Tissue procurement

Four primary LMS and 20 LMA were obtained from individual patients undergoing hysterectomy at University of Colorado Hospital (UCH) or the Gynecologic Tissue and Fluid Bank (GTFB), with Colorado Multiple Institutional Review Board approval (COMIRB 07–0935, 03–642 and 13–2003) from 2006 to 2012. Specimens were snap frozen in liquid nitrogen, and stored at −80 °C. Six formalin fixed paraffin embedded (FFPE) LMS samples from 2000 to 2014 were obtained from the UCH Pathology Department. In total, we evaluated 10 LMS (3 microarray and 7 independent samples) and 20 LMA (3 microarray and 17 independent samples). No STUMP lesions were included. All diagnoses were confirmed by a board certified gynecological pathologist.

2.2. Microarray analysis and gene selection

Preparation of 3 LMS, 3 LMA, and 3 normal myometrial fresh tissue samples was performed per protocol for the Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). Data were imported into GeneSpring (Agilent Technologies, Santa Clara, CA) and analyzed by GC-robust multiarray average, filtering, and ANOVA as described (Dimitrova et al., 2009) ($p < .05$, FDR 0.10) for 28,869 genes. Samples were grouped by tissue type revealing 777 statistically significant genes ($p < .05$) (Supp. 1). Of those, 378 were differentially expressed (fold change ≥1.5) in LMS and LMA as compared to normal myometrium (Supp. 2). A subset of 60 genes with functional roles in tumor progression and/or suppression was identified and seven genes with the greatest differential expression (fold change ≥2) and a documented role in gynecologic cancers, selected for confirmatory analysis.

2.3. RNA isolation

Tissue (1 g) from LMS and LMA was diced in 2 mL of Qiazol (Qiagen, Valencia, CA) and homogenized using the Polytron PT 2100 (Kinematica, Switzerland). Tissue was incubated in Qiazol for 15 min, 400 μL of chloroform was added, and RNA extracted per protocol using the miRNEasy Mini Kit (Qiagen). Two 10 μm FFPE LMS sections per sample were deparaffinized in heptane and methanol and RNA extracted per RNEasy FFPE Kit (Qiagen) protocol. RNA quality and concentration were assessed by on a RNA 6000 Nanochip using an Agilent 2100 bioanalyzer (Agilent Technologies).

2.4. cDNA synthesis

cDNA was generated from 1 μg total RNA using the QuantiTect RT Kit (Qiagen) with random primers per protocol.

2.5. qRT-PCR

Published primer sequences (Supp. 3) were synthesized (Life Technologies, Carlsbad, CA) or obtained commercially (Qiagen). qRT-PCR was performed using Sso Fast Evagreen Supermix (Bio-Rad, Hercules, CA) and analyzed in triplicate on a CFX96 Touch real-time PCR detection system (Bio-Rad): 95 °C for 30 s × 1, 95 °C then 60 °C for 5 s each x 40, 65–95 °C in 0.5 °C increments for 5 s each. PCR products were validated by melt curve and visualized by agarose gel electrophoresis. Genes were normalized to ubiquitin and relative expression calculated using the delta Ct method. Differences in expression were analyzed by *t*-test.

2.6. Immunohistochemistry

Paraffin sections (5 μm) were deparaffinized, antigens unmasked and immunohistochemically stained for CHI3L1 (Quidel, San Diego, CA; rabbit polyclonal; 1:200), MELK (Abcam, Cambridge, MA; rabbit polyclonal; 1:700), TOP2A (Abcam; rabbit monoclonal EP1102Y; 1:100), TPX2 (Abcam; rabbit polyclonal; 1:100), PRC1 (Santa Cruz Biotechnology, CA; rabbit polyclonal, 1:100), HPGD (Novus Biologicals, Littleton, CO; rabbit polyclonal; 1:1000) and TES (Abcam; mouse monoclonal 1G11-B7; 1:300). Equivalent concentrations of species and sub-class matched IgGs were used as negative controls. Antibodies were diluted in TBST +1% BSA *w/v* + 0.05% sodium azide. Antigens to PRC1 and TES were revealed in BORG solution (Biocare Medical, Concord, CA) and all others in 10 mM sodium citrate +0.1% tween, pH 6.0 for 10 min at 110 °C (NxGen Decloaking chamber, Biocare) with a 10 min ambient cool down. Immunodetection was performed on the Benchmark XT autostainer (Ventana, Tucson, AZ) at 37 °C. Primary antibodies were incubated for 32 min, except PRC1 for 40 min, and detected with the UltraView DAB universal polymer kit (Ventana). Sections were counterstained in Harris hematoxylin and mounted using

Table 1
Clinical characteristics of patients with primary uterine leiomyosarcoma.

Sample ID	Age	Menstrual status	[a]Stage	NAT	AT	First recurrence	Recurrence location	Vital status	[b]OS
LMS 1	62	PMP	IIIa	None	U	U	U	U	U
LMS 2	63	PMP	Ib	None	C	6 months	Lung	D	68
LMS 4	46	PRE	IIb	None	U	U	U	D	52
GTFB 538	55	PMP	IVb	C	C	-	-	D	48
S01	71	PMP	IIb	None	None	4 months	Pelvis	D	6
S02	75	PMP	Ib	None	R	None	None	D	64
S03Y	58	PMP	IIb	None	C	None	None	L	N/A
S04	67	PMP	IVb	None	U	U	U	D	8
S11	60	PMP	IIb	None	None	2 months	Pelvis	D	3
S14	63	PMP	Ib	None	None	None	None	L	N/A

NAT = neoadjuvant therapy, AT = adjuvant therapy, OS = overall survival, PMP = postmenopausal, PRE = premenopausal, R = radiation therapy, C = chemotherapy, U = unknown, D = deceased, L = living.
[a] FIGO 2009 staging classification.
[b] OS given in months.

synthetic resin. Scoring of staining intensity and distribution was performed by a gynecologic pathologist blinded to tumor type. Differences in expression were determined by *t*-test.

3. Results

3.1. Clinical parameters

We obtained de-identified clinical data for each patient with LMS (Table 1). The majority of patients were post-menopausal. Tumor stage varied significantly, however 70% presented with advanced disease (stage II or greater). Complete follow-up data were available for all but 3 patients. Overall survival (OS) ranges varied, but appeared at least partly dependent on receipt of adjuvant therapy. All but one patient (GTFB 538) underwent primary surgical therapy without neoadjuvant chemotherapy. GTFB 538 was included in the final analysis as, consistent with the literature, its expression profile appeared unaltered (Beck et al., 2010). No patients received hormonal therapy prior to surgery.

3.2. Microarray analysis of leiomyoma, leiomyosarcoma and normal myometrium

Our microarray analysis included 3 LMS, 3 LMA, and 3 normal myometrial tissue samples from individual patients. We included only biologic replicates based on excellent intra- and inter-laboratory correlations between technical replicates using the Affymetrix platform (Dobbin et al., 2005). Additionally, despite the heterogenous appearance of uterine LMS, tumor sampling location had minimal impact on gene expression profiles (Shmulevich et al., 2002).

Three hundred seventy-eight genes exhibited statistically significant differential expression (fold change ≥ 1.5) between uterine LMA and LMS as compared to normal myometrium (Fig. 1A). A subset of 60 differentially expressed genes with functional roles in tumor progression and suppression is also shown (Fig. 1B and Table 2). Consistent with prior analyses, over half of the genes overexpressed in LMS play a central role in cytokinesis, mitotic spindle assembly, or centrosome organization (Shan et al., 2012). These include: *TPX2*, *ANLN*, *ARHGAP11A*, *KIAA0101*, *MELK*, *CDC20*, *PRC1*, *ASPM*, *KPNA2*, *CENPF*, *NUF2*, *CHEK1*, *PLK1*, *KIF4A*, *FOXM1*, *RACGAP1*, *KIF23*, *KIF11*, *STIL*, *CENPK*, *CDK1*, *RRM1*, *ECT2*, *CDK2*, *STMN1*, and *BRCA2*. This further suggests a crucial role for the cytokinetic pathway in LMS progression, and provides a platform for development of molecularly targeted therapeutics.

3.3. mRNA expression analysis

Based on their published roles in gynecologic cancers, seven of the most differentially expressed genes between the tumor types, were selected for further evaluation (Table 3). As previously shown, many genes upregulated in LMS relative to normal myometrium are also upregulated compared to LMA (Skubitz and Skubitz, 2003). This gave us a proxy upon which to base direct comparison of uterine LMS and LMA and is also an important consideration in future development of a clinical assay, wherein tissue from indeterminate uterine mass(es) may also be compared to normal myometrium.

Statistically significant differences in mRNA expression were noted between LMS and LMA for each of the genes assayed. Consistent with our microarray, *CHI3L1*, *MELK*, *PRC1*, *TOP2A*, and *TPX2* mRNA were all overexpressed in LMS as compared to LMA (Figs. 2A-E). Conversely, *HPGD* and *TES* mRNA were overexpressed in LMA as compared to LMS (Figs. 2F-G). For genes overexpressed in LMS, we generally observed a wide variation in relative LMS mRNA expression, however very little variation in LMA. For genes overexpressed in LMA, variation in expression of LMS genes also appeared to contribute to lack of statistical significance between LMA vs. LMS-FFPE and vs. LMS-FRESH for *HPGD* and *TES,* respectively. We hypothesize that this could be due to normal expression variation within LMS. Additionally, RNA degradation in the LMS-FFPE samples could have influenced downstream PCR, particularly for HPGD. Nonetheless, a trend toward overexpression of *HPGD* and *TES* in LMA is demonstrated for those samples not reaching significance.

While we evaluated both fresh and FFPE LMS samples for each of the genes noted above via qRT-PCR, only 3 of 5 genes overexpressed in LMS were detected in FFPE specimens. *CHI3L1* and *TPX2* were observed only in fresh LMS samples (Fig. 2A and E). This may be due to low abundance of these gene transcripts and/or factors influencing RNA quality in FFPE samples (von Ahlfen et al., 2007). In Figs. 2B-D, we show that *MELK*, *PRC1* and *TOP2A* are overexpressed in LMS compared to LMA, with statistically significant differences in expression for both fresh and FFPE samples. While all 6 of the FFPE samples exhibited increased levels of *PRC1* and *TOP2A*, only 3 showed expression of *MELK*.

3.4. Immunohistochemical analysis of differentially expressed genes

For each gene of interest, we performed immunohistochemistry to evaluate differences in protein expression. CHI3L1, PRC1, TPX2, HPGD and TES were scored according to staining intensity. As the staining intensity of TOP2A was relatively uniform in each sample, it was scored based on percent positive cells. MELK staining was too variable to quantify reproducibly and not included in the analysis. CHI3L1, HPGD and TOP2A exhibited nuclear staining. PRC1 was predominantly nuclear with diffuse cytoplasmic staining and TPX2 and TES showed granular cytoplasmic staining (Fig. 3B). As shown (Fig. 3A), CHI3L1, PRC1, and TOP2A, exhibit greater protein expression in LMS as compared to LMA. In contrast, greater expression of HPGD in LMA is seen as

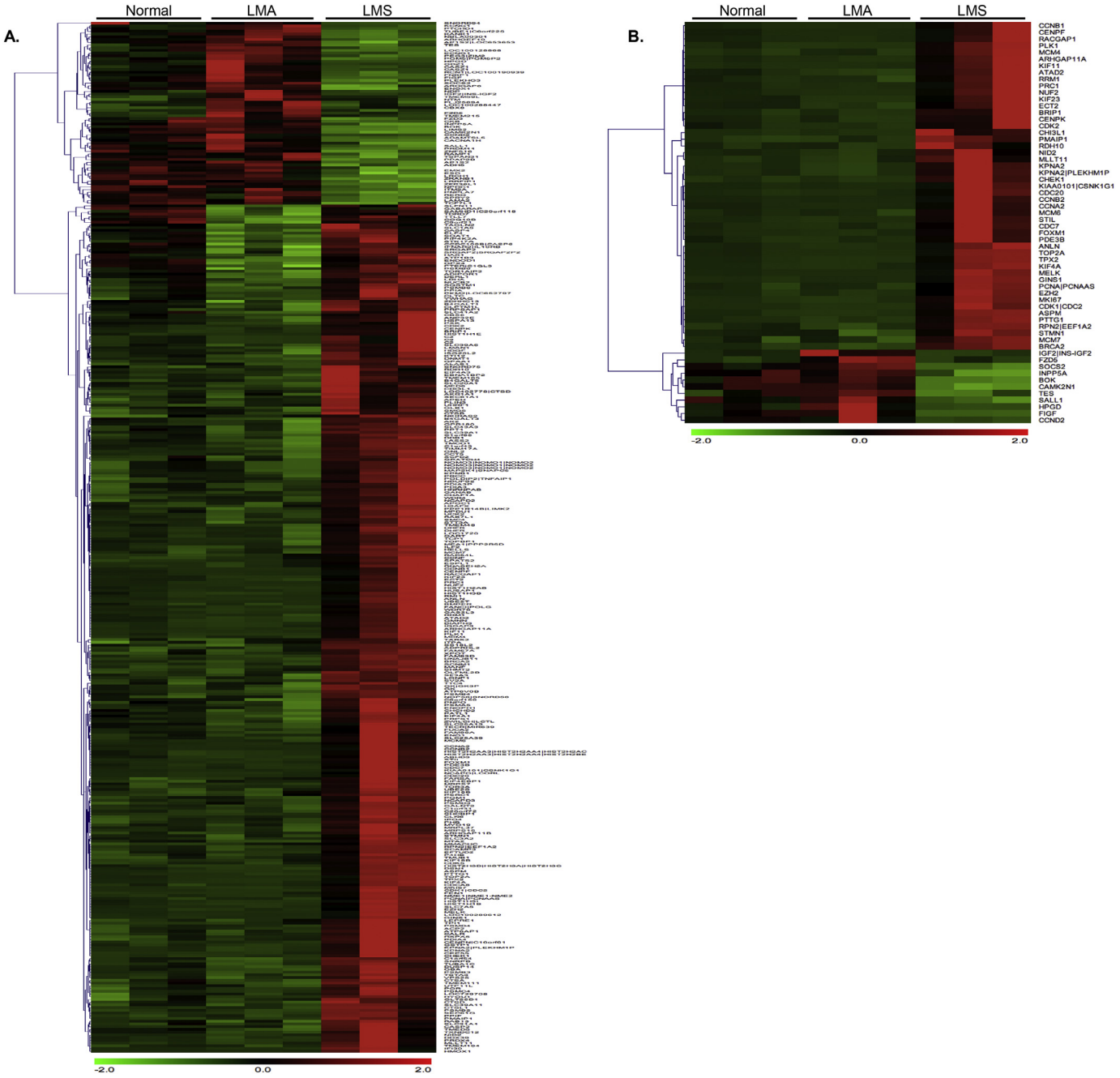

Fig. 1. Heatmap of statistically significant genes differentially expressed between uterine leiomyoma (LMA) and leiomyosarcoma (LMS) as compared to normal myometrium. Gene expression analysis performed on the Affymetrix Human Gene 1.0 ST array platform. (A) Genes with statistically significant (ANOVA) ≥1.5-fold differential expression [up-regulation (*red*) or down-regulation (*green*)] in normal myometrium, LMA and LMS (n = 378) are shown. (B) Genes with statistically significant (ANOVA) ≥1.5-fold differential expression in normal myometrium, LMA and LMS and functional roles in tumor progression or suppression (n = 60) are shown in a separate heatmap. Prior to hierarchical clustering, each gene was normalized to its average expression, such that the intensities center around 1 and are presented on a scale of −2 to +2. Fold changes and P values for these genes are listed in Supplementary Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to LMS. These each positively correlated with relative mRNA expression. There was no statistically significant difference between LMS and LMA TPX2 protein expression, and paradoxically, TES protein was more highly expressed in LMS. Overexpression of TES in LMS has been previously shown (Baird et al., 2005), however in comparison to LMA, the discrepancy remains unexplained. While this may reflect small sample size or tissue specific discordancy, other possibilities include context dependent expression and post-transcriptional or post-translational regulatory mechanisms. Representative histologic images are shown in Fig. 3B for each gene assayed. As seen, CHI3L1, PRC1, TOP2A, and TES proteins were more highly expressed in LMS, while HPGD was more strongly expressed in LMA. TPX2 showed similar LMS and LMA staining intensities.

4. Discussion

In this study we identify a novel gene expression signature with prominent potential to aid in distinguishing uterine LMS from LMA. Following microarray analysis, we selected 7 of the most differentially expressed genes between LMS and LMA with previously identified roles

Table 2
Genes differentially expressed between uterine leiomyosarcoma and leiomyoma with functional roles in tumor progression and suppression.

Entrez Gene ID	Gene symbol	Description	*LMS	*LMA	Putative Role
Upregulated in LMS					
1116	*CHI3L1*	chitinase 3-like 1 (cartilage glycoprotein-39)	18.246	−1.107	AA, G, M, P, T
7153	*TOP2A*	topoisomerase (DNA) II alpha 170 kDa	7.893	−1.762	CR, CS, P
22974	*TPX2*	targeting protein for Xklp2	6.704	−1.515	AA, CR, G, M, P, T
54443	*ANLN*	anillin, actin binding protein	6.049	−1.495	P, M
9824	*ARHGAP11A*	rho GTPase activating protein 11A	5.811	−1.229	A, AP, M
5366	*PMAIP1*	phorbol-12-myristate-13-acetate-induced protein 1, NOXA	5.777	1.130	CS, TS
9768|53944*	*KIAA0101|CSNK1G1*	KIAA0101 | casein kinase 1, gamma 1	5.694	−1.095	AP, CR, M, P
9833	*MELK*	maternal embryonic leucine zipper kinase	5.471	−1.188	AA, A, M, P, T
991	*CDC20*	cell division cycle 20 homolog (*S. cerevisiae*)	5.418	−1.032	AA, CR, P, T
9055	*PRC1*	protein regulator of cytokinesis 1	5.357	−1.396	P
259266	*ASPM*	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	5.257	-1.231	AA, M, P, T
3838	*KPNA2*	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	5.121	−1.486	AA, M, P, T
3838|440456*	*KPNA2|PLEKHM1P*	karyopherin alpha 2 (RAG cohort 1, importin alpha 1) | pleckstrin homology domain containing, family M (with RUN domain) member 1 pseudogene	5.086	−1.536	AA, M, P, T
1063	*CENPF*	centromere protein F, 350/400 ka (mitosin)	5.012	−1.406	M, P, T
9133	*CCNB2*	cyclin B2	4.946	−1.118	P
83540	*NUF2*	kinetochore Protein Nuf2 (NDC80 kinetochore complex component)	4.732	−1.307	AA, P
1111	*CHEK1*	CHK1 checkpoint homolog (S. pombe)	4.722	1.014	AA, CR, M, P, T
891	*CCNB1*	cyclin B1	4.654	−1.220	AA, M, P, T
157506	*RDH10*	retinol dehydrogenase 10 (all-trans)	4.622	−1.238	AP
5347	*PLK1*	polo-like kinase 1 (Drosophila)	4.608	−1.167	AA, CR, G, M, P, T
9837	*GINS1*	GINS complex subunit 1 (Psf1 homolog)	4.487	1.032	AA, M, P, T
22795	*NID2*	nidogen 2 (osteonidogen)	4.414	−1.452	CA
24137	*KIF4A*	kinesin family member 4A	4.376	−1.113	AP, M, P, TS
4288	*MKI67*	antigen identified by monoclonal antibody Ki-67	4.327	−1.154	P
10,962	*MLLT11*	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11	4.228	−1.090	A, CR, CS, M, P, T
83990	*BRIP1*	BRCA1 interacting protein C-terminal helicase 1	3.870	−1.069	TS
2305	*FOXM1*	forkhead box M1	3.824	−1.097	AA, CR, G, M, P, T
890	*CCNA2*	cyclin A2	3.773	−1.230	AA, CA, CR, P
4173	*MCM4*	minichromosome maintenance complex component 4	3.772	1.082	P
29127	*RACGAP1*	rac GTPase activating protein 1	3.705	−1.200	AA, CR, M, P
9493	*KIF23*	kinesin family member 23	3.686	−1.615	AA, P, T
3832	*KIF11*	kinesin family member 11	3.524	−1.037	AA, G, M, P, T
29028	*ATAD2*	ATPase family, AAA domain containing 2	3.523	−1.725	AA, M, P, T
5111|100302739**	*PCNA|PCNAAS*	proliferating cell nuclear antigen | PCNA antisense RNA (non-protein coding)	3.490	1.028	P, T
9232	*PTTG1*	pituitary tumor-transforming 1	3.423	−1.159	A, AA, G, M, P, T
2146	*EZH2*	enhancer of zeste homolog 2 (Drosophila)	3.338	−1.023	AA, CR, G, M, P, T
8317	*CDC7*	cell division cycle 7 homolog (S. cerevisiae)	3.268	−1.054	AA, CR, P, T
4175	*MCM6*	minichromosome maintenance complex component 6	3.108	−1.133	P
6491	*STIL*	SCL/TAL1 interrupting locus	3.049	−1.085	AA, G, P, T
64105	*CENPK*	centromere protein K	3.025	−1.169	P
983**	*CDK1|CDC2*	cyclin-dependent kinase 1 | cell division cycle 2, G1 to S and G2 to M	2.882	−1.085	AA, M, P, T
6240	*RRM1*	ribonucleotide reductase M1	2.846	−1.228	A, AP, CA, CR, M, P, TS
1894	*ECT2*	epithelial cell transforming sequence 2 oncogene	2.683	−1.437	AA, M, P, T
5140	*PDE3B*	phosphodiesterase 3B, cGMP-inhibited	2.663	−1.083	AA, CR, P
6185|1917**	*RPN2|EEF1A2*	ribophorin II | eukaryotic translation elongation factor 1 alpha 2	2.433	−1.232	AA, CR^, G§, M, P, T
1017	*CDK2*	cyclin-dependent kinase 2	2.217	−1.154	A, AA, CR, G, M, P, T
3925	*STMN1*	stathmin 1	2.129	−1.378	AA, CR, M, P, T
4176	*MCM7*	minichromosome maintenance complex component 7	2.066	1.005	AA, P, M, T
675	*BRCA2*	breast cancer 2, early onset	1.711	−1.065	CS, TS
Upregulated in LMA					
7855	*FZD5*	frizzled homolog 5 (Drosophila)	−1.035	4.690	CA, G, M, P
3481|723961*	*IGF2|INS-IGF2*	insulin-like growth factor 2 (somatomedin A) | INS-IGF2 readthrough transcript	−1.564	3.658	AA, CR, G, M, P, T
3248	*HPGD*	hydroxyprostaglandin dehydrogenase 15-(NAD)	−3.168	2.385	TS
26136	*TES*	testis derived transcript (3 LIM domains)	−4.330	2.077	TS
8835	*SOCS2*	suppressor of cytokine signaling 2	−2.622	1.563	AA, P, TS
2277	*FIGF*	c-fos induced growth factor (vascular endothelial growth factor D)	−2.567	1.461	AA, G, M
6299	*SALL1*	sal-like 1 (Drosophila)	−2.116	1.253	TS
894	*CCND2*	cyclin D2	−7.864	1.216	P
3632	*INPP5A*	inositol polyphosphate-5-phosphatase, 40 kDa	−2.668	1.147	TS
666	*BOK*	BCL2-related ovarian killer	−2.094	1.092	A
55450	*CAMK2N1*	calcium/calmodulin-dependent protein kinase II inhibitor 1	−4.148	1.066	TS

A = apoptotic, AA = antiapoptotic, AP = antiproliferative, CA = cell adhesion, CR = chemoresistance, CS = chemosensitivity, G = angiogenesis, M = metastasis or invasion, P = proliferative, T = tumorigenic, TS = tumor suppressor.
* Putative role applies only to primary gene.
**Putative role includes readthrough sequence.
^ = RPN2 only, § = EEF1A2 only.
*Values represent expression fold change as compared to normal myometrium.

Table 3
Selected genes differentially expressed between uterine leiomyosarcoma and leiomyoma with relevance to gynecologic cancers.

Gene Symbol	Gene Name	[a]LMS	[a]LMA	Upregulated
CHI3L1	Chitinase 3-like 1	18.246	−1.107	LMS
TOP2A	Topoisomerase II alpha	7.893	−1.762	LMS
TPX2	Targeting protein for Xklp2	6.704	−1.515	LMS
MELK	Maternal embryonic leucine zipper kinase	5.471	−1.188	LMS
PRC1	Protein regulator of cytokinesis 1	5.357	−1.396	LMS
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	−3.168	2.385	LMA
TES	Testin LIM domain protein	−4.330	2.077	LMA

LMS = leiomyosarcoma, LMA = leiomyoma.
[a] Values represent expression fold change as compared to normal myometrium.

in tumor promotion or suppression and gynecologic cancer relevance. This allowed us to identify putative targets for prognostic and therapeutic application in the treatment of LMS.

Chitinase 3-like 1 (CHI3L1) is a secreted glycoprotein that promotes invasion and angiogenesis, while inhibiting apoptosis. Elevation of CHI3L1 and poor prognosis have been observed in patients with breast, glioma, renal, prostate, colorectal, and ovarian cancers (Jensen et al., 2003). *CHI3L1* was previously identified as one of the most highly overexpressed genes in LMS (Matsumura et al., 2006). Maternal embryonic leucine zipper kinase (MELK) regulates cell cycle and apoptosis. It is associated with higher tumor grade and decreased survival in brain and breast cancers, respectively. MELK is upregulated in serous endometrial cancer and synovial sarcomas (Risinger et al., 2013). Protein regulator of cytokinesis 1 (PRC1) associates with mitotic spindles. It is upregulated in fibrosarcomas (Baird et al., 2005), invasive cervical, and breast cancers (Shimo et al., 2007). PRC1 is overexpressed in LMS (Shan et al., 2012; Skubitz and Skubitz, 2003). Topoisomerase II alpha (TOP2A) relieves torsional stress during DNA transcription and replication. TOP2A expression has prognostic significance in breast, prostate, and ovarian cancers (de Resende et al., 2013). It is also upregulated in LMS (Shan et al., 2012; Skubitz and Skubitz, 2003). Targeting protein for Xklp2 (TPX2), associates with Aurora kinase A, and mediates proliferation via mitotic spindle assembly. It is overexpressed in high grade in cervical carcinoma and associated with lower disease free survival in high-grade serous ovarian cancer (Chang et al., 2012).

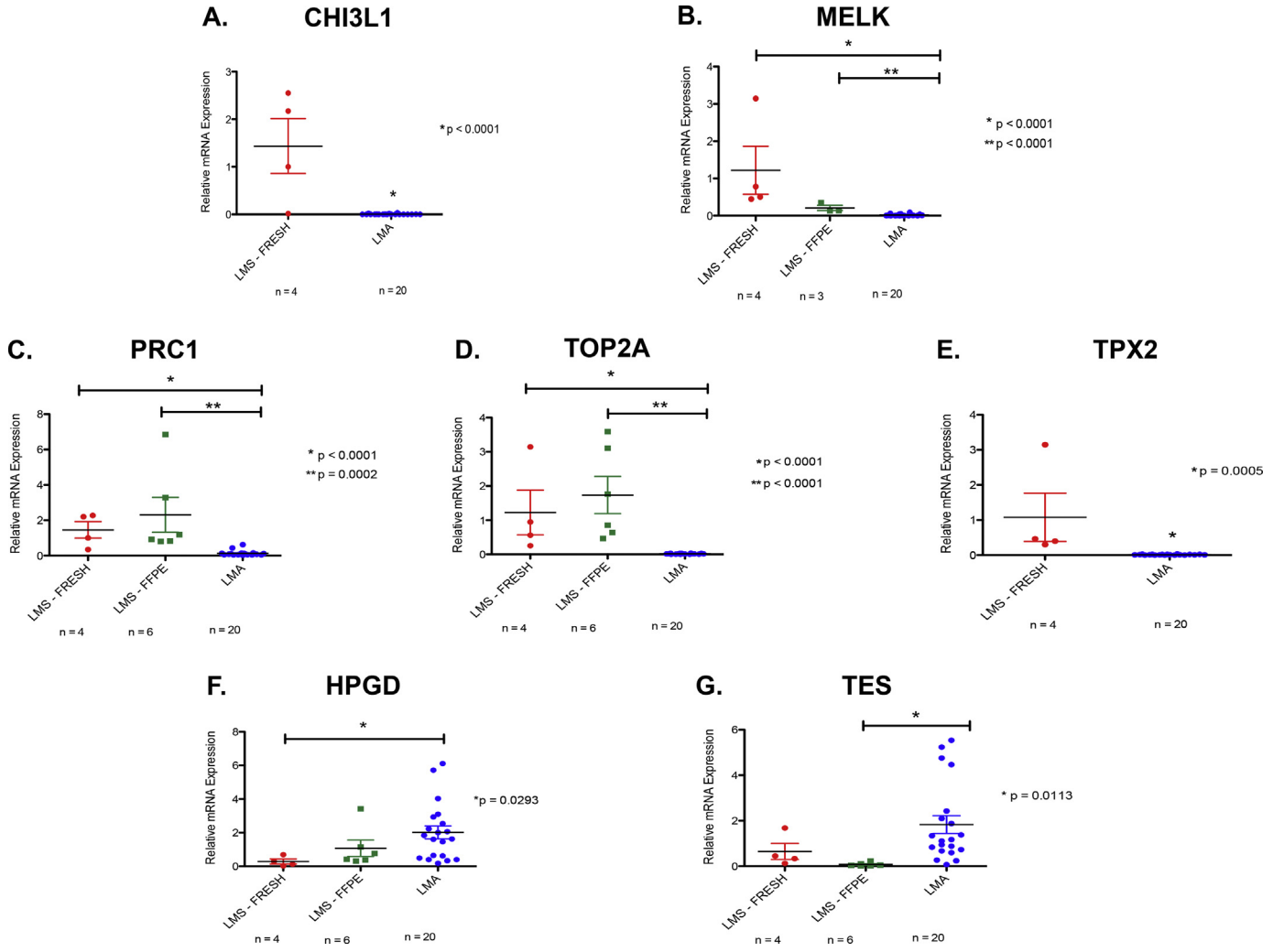

Fig. 2. *CHI3L1*, *MELK*, *PRC1*, *TOP2A*, *TPX2*, *HPGD* and *TES* mRNA expression in uterine leiomyosarcomas and leiomyomas. Semiquantitative RT-PCR was performed to assess relative mRNA expression in uterine leiomyosarcomas and leiomyomas. CHI3L1, MELK, PRC1, TOP2A, and TPX2 (A-E) are overexpressed in leiomyosarcomas (LMS – FRESH and LMS - FFPE) as compared to leiomyomas (LMA). HPGD and TES (F-G) are overexpressed in LMA, as compared to LMS – FRESH and LMS-FFPE, respectively. No transcript expression from the paraffin embedded LMS specimens was observed for CHI3L1 or TPX2 (A and E). Mean relative mRNA expression levels as indicated for each tumor type: LMS fresh – red dots, LMS FFPE – green squares, LMA – blue dots. Error bars indicate mean ± SEM. * = $p < .05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

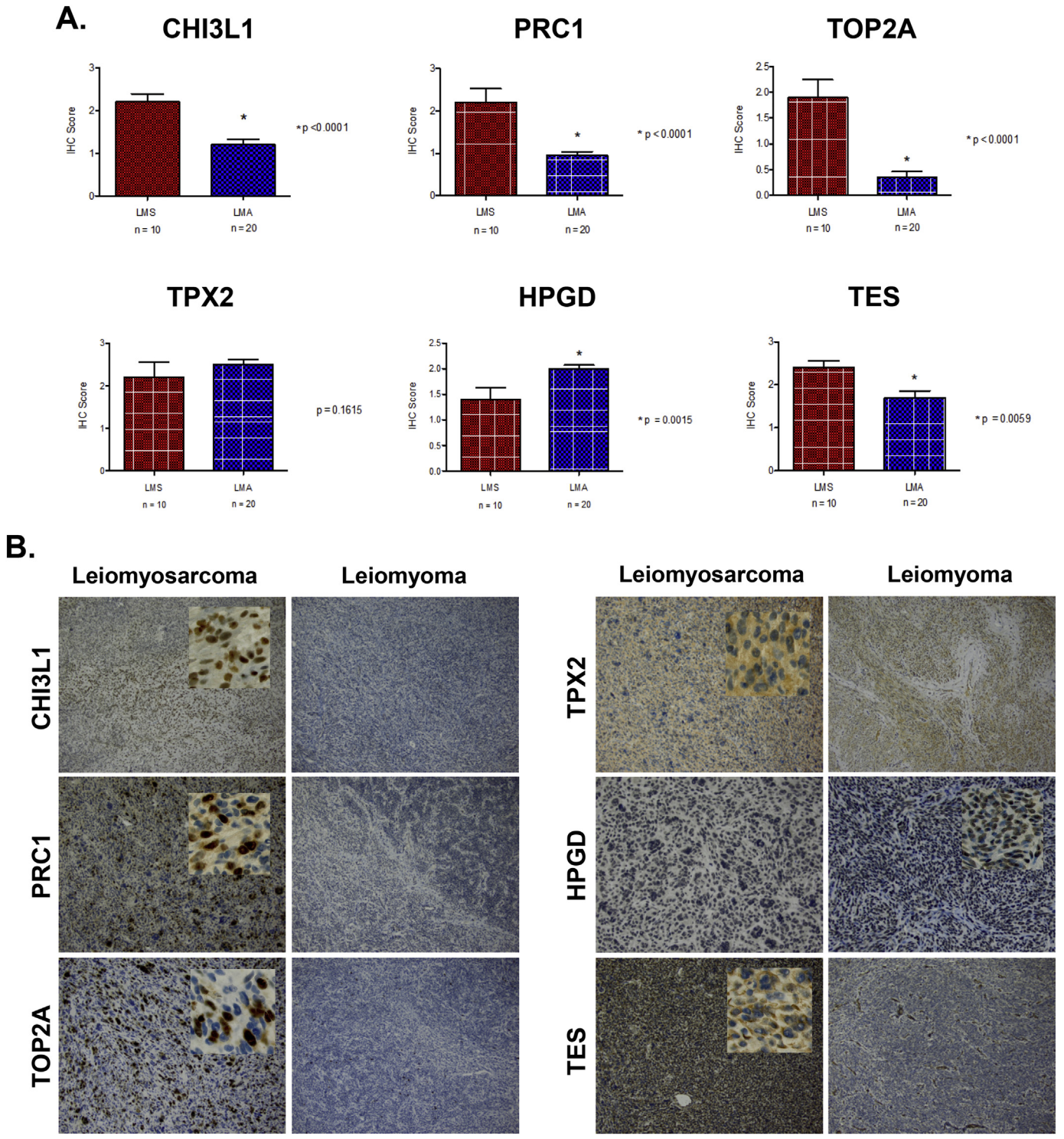

Fig. 3. CHI3L1, PRC1, TOP2A, TPX2, HPGD and TES protein expression and immunohistochemical staining in uterine leiomyosarcomas and leiomyomas. (A) Immunohistochemical grading was performed to assess protein expression in uterine leiomyosarcomas (LMS) and leiomyomas (LMA). Images were analyzed for staining intensity: 0 = none, 1 = focal or faint and rare, 2 = moderate or faint but diffuse, 3 = strong, or percentage of positive cells (TOP2A only): 0 ≤ 1%, 1 = 1–10%, 2 = 11–50%, and 3 ≥ 50%. CHI3L1, PRC1, TOP2A, and TES proteins are more highly expressed in LMS, while HPGD is more highly expressed in LMA. No significant difference in TPX2 protein expression was noted between the tumor types. Relative levels of protein expression as indicated by mean IHC score are noted for each tumor type: LMS fresh and FFPE – red bar, LMA – blue bar. Protein expression was concordant with mRNA expression for each gene except TPX2 and TES. Error bars indicate mean ± SEM. * = $p < .05$. (B) Representative immunohistochemical staining of CHI3L1, PRC1, TOP2A, TPX2, HPGD, and TES proteins in leiomyosarcoma (left) and leiomyoma (right). MELK staining (not shown) was too variable to quantify accurately. Images are 10× (except for HPGD 20×). Inserts show 40× high power views of positive staining LMS or LMA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TPX2 and Aurora A are also overexpressed in LMS (Shan et al., 2012). Hydroxyprostaglandin dehydrogenase 15-(NAD), HPGD, is responsible for prostaglandin degradation. It functions as a tumor suppressor in colon and breast cancers (Wolf et al., 2006). Additionally, HPGD is downregulated in cervical cancer (Wong et al., 2006). The scaffolding protein testin (TES) mediates interactions between transcription factors and signaling proteins. It is a tumor suppressor with prognostic implications in gastric, uterine, breast, and ovarian cancers (Drusco et al., 2005). Restoration of TES expression in MES-SA uterine sarcoma cells results in growth suppression (Sarti et al., 2005), however TES may be overexpressed in LMS tumors (Baird et al., 2005).

We demonstrate overexpression of *CHI3L1*, *MELK*, *PRC1*, *TOP2A*, and *TPX2* in LMS compared to LMA, and overexpression of *HPGD* and *TES* in LMA relative to LMS. Although the number of tissue samples assessed in the study was small, we were able to corroborate the findings of others with regard to expression of *CHI3L1*, *PRC1*, *TOP2A*, and *TPX2* (Shan et al., 2012; Skubitz and Skubitz, 2003). We also identified novel differential expression of *MELK* and *HPGD* in LMS and LMA. In contrast to other analyses, we utilized qRT-PCR and IHC for more complete validation of this unique gene expression profile. High throughput analyses have confirmed gene expression differences between sarcomas of multiple origins (Baird et al., 2005) with important prognostic implications (Rao et al., 1999). Thus, inclusion of uterine tumors exclusively, may improve tissue-specific application of this gene panel.

Advantages of using qRT-PCR to aid in differentiation of these unique tumors, include faster processing, easier sample preparation and use of smaller tissue samples. qRT-PCR is useful when a reliable antibody is unavailable, and may also clarify uncertain IHC results. Additionally, qRT-PCR can be performed on FFPE LMS samples, making this a potentially useful modality for diagnostic confirmation in tertiary care centers, wherein no fresh tissue is available.

We note that gene expression differences exist between uterine LMA with and without del (7q), particularly 7q22.2-q22.3, and may impact diagnostic accuracy in the setting of future gene panel expansion. However, with the exception of TES (7q31.2), none of the genes in our panel were located on chromosome 7, and no significant LMA expression differences were attributable to the 7q genotype (Hodge et al., 2009).

There are currently no reliable serum biomarkers to diagnose or distinguish LMA from LMS (Babacan et al., 2014; Levy et al., 2013). The molecular signature observed in this study may aid both pre- and postoperative diagnostic evaluation, with potential benefits of risk stratification for those considering a minimally invasive hysterectomy and more rapid diagnosis in cases with unclear tumor histology and etiology. In the preoperative setting, we propose that hysteroscopically-guided biopsy is feasible for tumors with a significant intracavitary component. This gene expression panel may also have diagnostic and prognostic significance in atypical LMA and smooth muscle tumors of uncertain malignant potential. Future studies will increase sample size to further define and validate gene expression differences between uterine LMS and LMA.

In summary, uterine LMS presents a unique diagnostic and therapeutic challenge. Lack of distinguishing symptoms, exam findings or diagnostic tests, and shared histologic abnormalities significantly limit our ability to clinically differentiate these tumors from benign uterine LMA. Additionally, therapeutic options for LMS are limited and the prognosis is poor regardless of stage, further demonstrating the need for improved molecular characterization of this aggressive cancer. In this study, we present a unique gene expression signature, which may expand our ability to promptly and accurately differentiate between uterine LMS and LMA and guide treatment. In addition to expanding our diagnostic capabilities, it may also enhance our understanding of potential prognostic factors and provide novel therapeutic targets for the treatment of LMS.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Funding

This work was made possible by NIH 5K12HD00127–15 Women's Reproductive Health Research Career Development Program, CU Department of Obstetrics and Gynecology Academic Enrichment Fund, CU Cancer Center Cancer Research Summer Fellowship Program, CU Cancer Center Cocktails for a Cure, and the Leiomyosarcoma Research Fund donated by the friends and family of Tina Carlson.

Acknowledgements

Affymetrix array analysis was performed through the University of Colorado Gene Expression Core. The authors appreciate the contribution to this research made by E. Erin Smith, HTL(ASCP)^{CM}QIHC of the University of Colorado Denver Research Histology Shared Resource. This resource is supported in part by the Cancer Center Support Grant (P30CA046934). We would like to thank Annette Joglar, BS and the University of Colorado GTFB for kindly contributing rare LMS tissue samples. We would also like to thank Heidi Wilson, PhD for critical review and revision of the manuscript. Contents are the authors' sole responsibility.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.104284.

References

Amant, F., et al., 2009. Clinical management of uterine sarcomas. Lancet Oncol. 10, 1188–1198.
Babacan, A., et al., 2014. CA 125 and other tumor markers in uterine leiomyomas and their association with lesion characteristics. Int. J. Clin. Exp. Med. 7, 1078–1083.
Baird, D.D., et al., 2003. High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. Am. J. Obstet. Gynecol. 188, 100–107.
Baird, K., et al., 2005. Gene expression profiling of human sarcomas: insights into sarcoma biology. Cancer Res. 65, 9226–9235.
Bansal, N., et al., 2008. The utility of preoperative endometrial sampling for the detection of uterine sarcomas. Gynecol. Oncol. 110, 43–48.
Beck, A.H., et al., 2010. Discovery of molecular subtypes in leiomyosarcoma through integrative molecular profiling. Oncogene. 29, 845–854.
Bogani, G., et al., 2015 Apr. Impact of morcellation on survival outcomes of patients with unexpected uterine leiomyosarcoma: a systematic review and meta-analysis. Gynecol. Oncol. 137 (1), 167–172. https://doi.org/10.1016/j.ygyno.2014.11.011. Epub 2014 Nov 20.
Brooks, S.E., et al., 2004. Surveillance, epidemiology, and end results analysis of 2677 cases of uterine sarcoma 1989-1999. Gynecol. Oncol. 93, 204–208.
Center for Devices and Radiological Health, 2014. Safety Communications. UPDATED Laparoscopic Uterine Power Morcellation in Hysterectomy and Myomectomy. FDA Safety Communication.
Chang, H., et al., 2012. The TPX2 gene is a promising diagnostic and therapeutic target for cervical cancer. Oncol. Rep. 27, 1353–1359.
de Resende, M.F., et al., 2013. Prognostication of prostate cancer based on TOP2A protein and gene assessment: TOP2A in prostate cancer. J. Transl. Med. 11, 36.
Dimitrova, I.K., et al., 2009. Gene expression profiling of multiple leiomyomata uteri and matched normal tissue from a single patient. Fertil. Steril. 91, 2650–2663.
Dobbin, K.K., et al., 2005. Interlaboratory comparability study of cancer gene expression analysis using oligonucleotide microarrays. Clin. Cancer Res. 11, 565–572.
Drusco, A., et al., 2005. Knockout mice reveal a tumor suppressor function for Testin. Proc. Natl. Acad. Sci. U. S. A. 102, 10947–10951.
Echt, G., et al., 1990. Treatment of uterine sarcomas. Cancer. 66, 35–39.
Farquhar, C.M., Steiner, C.A., 2002. Hysterectomy rates in the United States 1990-1997. Obstet. Gynecol. 99, 229–234.
Hodge, J.C., et al., 2009. Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosom. Cancer. 48, 865–885.
Jensen, B.V., et al., 2003. High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. Clin. Cancer Res. 9, 4423–4434.
Kapp, D.S., et al., 2008. Prognostic factors and survival in 1396 patients with uterine leiomyosarcomas: emphasis on impact of lymphadenectomy and oophorectomy. Cancer. 112, 820–830.
Kempson, R.L., Hendrickson, M.R., 2000. Smooth muscle, endometrial stromal, and mixed Mullerian tumors of the uterus. Mod. Pathol. 13, 328–342.
Lee, Y.F., et al., 2004. A gene expression signature associated with metastatic outcome in

human leiomyosarcomas. Cancer Res. 64, 7201–7204.
Lee, C.H., et al., 2009. A panel of antibodies to determine site of origin and malignancy in smooth muscle tumors. Mod. Pathol. 22, 1519–1531.
Levy, G., et al., 2013. Biomarkers in uterine leiomyoma. Fertil. Steril. 99, 1146–1152.
Mahnert, N., et al., 2015. Unexpected gynecologic malignancy diagnosed after hysterectomy performed for benign indications. Obstet. Gynecol. 125, 397–405.
Matsumura, N., et al., 2006. Oncogenic property of acrogranin in human uterine leiomyosarcoma: direct evidence of genetic contribution in in vivo tumorigenesis. Clin. Cancer Res. 12, 1402–1411.
Miyajima, K., et al., 2001. Relative quantitation of p53 and MDM2 gene expression in leiomyosarcoma; real-time semi-quantitative reverse transcription-polymerase chain reaction. Cancer Lett. 164, 177–188.
Quade, B.J., et al., 2004. Molecular pathogenesis of uterine smooth muscle tumors from transcriptional profiling. Genes Chromosom. Cancer. 40, 97–108.
Rao, U.N., et al., 1999. Comparative immunohistochemical and molecular analysis of uterine and extrauterine leiomyosarcomas. Mod. Pathol. 12, 1001–1009.
Ricci, S., et al., 2017. Uterine leiomyosarcoma: epidemiology, contemporary treatment strategies and the impact of uterine morcellation. Gynecol. Oncol. 145, 208–216.
Risinger, J.I., et al., 2013. Gene expression analysis of early stage endometrial cancers reveals unique transcripts associated with grade and histology but not depth of invasion. Front. Oncol. 3, 139.
Sarti, M., et al., 2005. Adenoviral transduction of TESTIN gene into breast and uterine cancer cell lines promotes apoptosis and tumor reduction in vivo. Clin. Cancer Res. 11, 806–813.
Shan, W., et al., 2012. A small-molecule inhibitor targeting the mitotic spindle checkpoint impairs the growth of uterine leiomyosarcoma. Clin. Cancer Res. 18, 3352–3365.
Shimo, A., et al., 2007. Elevated expression of protein regulator of cytokinesis 1, involved in the growth of breast cancer cells. Cancer Sci. 98, 174–181.
Shmulevich, I., et al., 2002. Tumor specific gene expression profiles in human leiomyosarcoma: an evaluation of intratumor heterogeneity. Cancer. 94, 2069–2075.
Skubitz, K.M., Skubitz, A.P., 2003. Differential gene expression in leiomyosarcoma. Cancer. 98, 1029–1038.
von Ahlfen, S., et al., 2007. Determinants of RNA quality from FFPE samples. PLoS One 2, e1261.
Wolf, I., et al., 2006. 15-hydroxyprostaglandin dehydrogenase is a tumor suppressor of human breast cancer. Cancer Res. 66, 7818–7823.
Wong, Y.F., et al., 2006. Genome-wide gene expression profiling of cervical cancer in Hong Kong women by oligonucleotide microarray. Int. J. Cancer 118, 2461–2469.
Worldwide, A. A. M. I. G, 2014. AAGL practice report: Morcellation during uterine tissue extraction. J. Minim. Invasive Gynecol. 21, 517–530.