GYNECOLOGY

The differential diagnoses of uterine leiomyomas and leiomyosarcomas using DNA and RNA sequencing

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BACKGROUND: Although uterine leiomyomas and leiomyosarcomas are considered biologically unrelated tumors, they share morphologic and histologic characteristics that complicate their differential diagnosis. The long-term therapeutic option for leiomyoma is laparoscopic myomectomy with morcellation, particularly for patients who wish to preserve their fertility. However, because of the potential dissemination of undiagnosed or hidden leiomyosarcoma from morcellation, there is a need to develop a preoperative assessment of malignancy risk.

OBJECTIVE: Through an integrated comparative genomic and transcriptomic analysis, we aim to identify differential genetic targets in leiomyomas vs leiomyosarcomas using next-generation sequencing as the first step toward preoperative differential diagnosis.

STUDY DESIGN: Targeted sequencing of DNA and RNA coding regions for solid tumor—associated genes was performed on formalin-fixed paraffin-embedded samples from 13 leiomyomas and 13 leiomyosarcoma cases. DNA sequencing was used to identify copy number variations, single-nucleotide variants, and small insertions/deletions. RNA sequencing was used to identify gene fusions, splice variants, and/or differential gene expression profiles.

RESULTS: In leiomyosarcomas, tumor mutation burden was higher in terms of copy number variations, single nucleotide variants, small insertions/deletions, and gene fusions compared with leiomyomas. For copy number variations, 20 genes were affected by deletions in leiomyosarcomas, compared with 6 observed losses in leiomyomas. Gains (duplications) were identified in 19 genes in leiomyosarcomas, but only 3 genes in leiomyomas. The most common mutations (single-nucleotide variants and insertions/deletions) for leiomyosarcomas were identified in 105 genes of all analyzed leiomyosarcomas; 82 genes were affected in leiomyomas. Of note, 1 tumor previously diagnosed as leiomyosarcoma was established as inflammatory myofibroblastic tumor along this study with a novel ALK-TNS1 fusion. Finally, a differential transcriptomic profile was observed for 11 of 55 genes analyzed in leiomyosarcomas; 8.5% of initially diagnosed leiomyosarcomas showed high-confidence, novel gene fusions that were associated with these tumors.

CONCLUSION: Through integrated comparative genomic and transcriptomic analyses, we identified novel differential genetic targets that potentially differentiate leiomyosarcomas and leiomyomas. This provides a new insight into the differential diagnosis of these myometrial tumors.

Key words: *BRCA2,* DNA/RNA sequencing, *FGFR4,* genomic/transcriptomic profile, *ROS1,* uterine leiomyoma, uterine leiomyosarcoma

Uterine leiomyomas are benign smooth muscle tumors with an estimated lifetime risk of approximately 70% of women of reproductive age.[1] These tumors produce complications that include pelvic pain, heavy menstrual bleeding, anemia, infertility, and recurrent pregnancy loss.[2,3] Although selective progesterone receptor modulators are used to manage leiomyoma,[4,5] surgery is the gold standard intervention and remains as the long-term therapeutic option. Specifically, laparoscopic myomectomy with morcellation is considered the best choice because it is less invasive, particularly for women who wish to preserve their fertility.[6] However, this surgery carries potential detrimental effects for patients with undiagnosed occult leiomyosarcoma.[7]

Leiomyosarcoma represents 70% of all uterine sarcomas but remains rare, with an incidence of 0.4—0.9 in 100,000 women.[8] They are aggressive malignant tumors that arise from the myometrium and that are characterized by early metastasis, poor prognosis, and high rates of recurrence with limited therapeutic efficacy.[9—11] The risk of occult uterine cancer in women with benign lesions is 1 in 350, and clinical symptoms and morphologic features between leiomyoma and leiomyosarcoma are indistinguishable.[12—17] Therefore, there is a risk of hidden malignancy during surgery.

Initial evidence that supports the risk of morcellation to spread hidden malignant tumors was publicized with the case of Dr Amy Reed. She underwent a total hysterectomy by laparoscopy with power morcellation for leiomyoma in 2013, but she later received a diagnosis of advanced stage 4 leiomyosarcoma, which resulted in her death in 2017.[18—20] In 2014, the Food and Drug Administration issued a press release discouraging the use of power morcellators to treat myometrial tumors.[21]

Researchers have since attempted to develop preoperative diagnostic tests to discriminate between benign and malignant uterine masses.[22] However, no clear evidence indicates that vaginal ultrasound and elastography[23,24] or magnetic resonance imaging and computed tomography[10,25] can discriminate leiomyoma and leiomyosarcoma. Further, observations of a differential protein

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AJOG at a Glance

Why was this study conducted?

Preoperative assessment of malignancy risk for uterine leiomyomas and leiomyosarcomas is needed to prevent accidental dissemination of cancer from morcellation.

Key Findings

Next-generation sequencing technologies identified novel differential genetic targets that may be used as future diagnostic markers for uterine leiomyomas and leiomyosarcomas.

What does this add to what is known?

Integrative molecular analysis of transcriptomic and genomic data can improve our understanding of the pathogenesis of these uterine tumors and provide a first step toward preoperative diagnosis of leiomyomas and leiomyosarcomas through liquid biopsy.

pattern are hampered by false-positive findings.[26]

At a molecular level, myometrial tumors show unbalanced karyotypes and nonspecific and complex alterations, such as single nucleotide variants, small insertions and deletions, gene amplifications, and gene fusions. High-throughput sequencing studies identified recurrent and mutually exclusive mutations among them.[27–29] The main drivers for change in leiomyoma are rearrangements in the *HMGA2*, mutations in *MED12*, *COL4A5–COL4A6* deletions, and biallelic inactivation of FH.[30,31] The most frequently mutated genes in leiomyosarcoma are *ATRX*, *PTEN*, *VIPR2*, *YWHAE*, and *TP53*.[32,33] However, some of these genes are not specific for smooth muscle malignancies, and the molecular mechanisms underlying tumorigenesis remain elusive.

Lack of an accurate preoperative or intraoperative diagnostic to differentiate myometrial tumors affect their surgical treatment. Food and Drug Administration regulation has substituted laparoscopic myomectomy for laparotomy-based procedures, increasing morbidity and mortality rates and the cost for the patient and healthcare system.[34] Here, we sought to investigate the existence of consistent differential genetic alterations at the genomic and transcriptomic levels between leiomyosarcoma and leiomyoma to establish an early differential diagnosis.

Materials and Methods

Detailed descriptions of the materials and methods used in this study are provided in the Supplemental Material. A total of 13 leiomyoma and 13 leiomyosarcoma from formalin-fixed paraffin-embedded (FFPE) samples were selected, processed, and confirmed histologically, according to World Health Organization criteria.[35,36] Of note, 1 of the initially diagnosed leiomyosarcomas was confirmed as inflammatory myofibroblastic tumor (IMT; sample IMT01) after the molecular analysis and subsequent histologic validation. The research was approved by the Institutional Review Board of University Hospital La Fe (2016/0118).

Illumina TruSight Tumor 170 kit (Illumina, San Diego, CA) for solid tumor-associated genes was used for targeted sequencing of DNA (including 148 single nucleotide variants and indels, and 59 amplification) and RNA coding regions (including 55 genes for fusions and splice variants). Bioinformatic analysis for small variants, including point mutations and indels, was performed by Pisces.[37] Variants with an allele frequency >1% in ExAC Browser or >1% in gnomAD Browser were filtered out. We retained variants with variant fraction >0.2 and coverage >6. Copy number variants (CNVs) were detected by Copy Number Variant Robust Analysis for Tumors (http://support.illumina.com/help/BS_App_TruSightTumor170_OLH_1000000028435/Content/Source/HomePages/Home_Page_TSight_Tumor170_App.htm). Additionally, RNA Splice Variant Caller software (https://support.illumina.com/help/BS_App_TruSightTumor170_OLH_1000000028435/Content/Source/HomePages/Home_Page_TSight_Tumor170_App.htm) was used for splice variant calling, and differential expression analysis was performed with the edgeR package[38] from Bioconductor software repository (https://bioconductor.org/packages/release/bioc/html/edgeR.html).[39] Last, fusion genes were identified with Manta RNA Fusion Calling software (https://github.com/Illumina/manta) and validated by immunohistochemistry and fluorescence in situ hybridization (FISH).

Results

Patient characteristics

Patients with a leiomyoma diagnosis had a median age of 43 years (range, 30–48 years); patients with a leiomyosarcoma diagnosis had a median age of 55 years (range, 44–67 years). All tumors were collected during primary resection, and 50% of leiomyosarcoma tumors were high-grade. Tumor size varied from 12–150 mm (median, 71.6±9.4 mm) in leiomyoma and 80–230 mm (median, 160±32.9 mm) in leiomyosarcoma (Table S1). Histologic information estimated approximately 69% of necrosis in leiomyosarcoma samples and approximately 78% with high mitotic activity (Table S2).

Comparative genomic analysis of leiomyoma and leiomyosarcoma

We comparatively screened for somatic mutations between leiomyoma and leiomyosarcoma samples. Average coverage reached a mean depth of 3535x, with a minimum coverage of 6 reads. An average of 20 mutations in 82 genes in leiomyoma and 22 mutations in 105 genes in leiomyosarcoma samples were observed (Table S3). The leiomyoma group represented approximately 3% of deletions, approximately 9% of insertions, and approximately 88% of single nucleotide polymorphisms; in leiomyosarcoma approximately 5% were deletions, approximately 9% insertions, and approximately 86% single

TABLE 1
Unique variants in leiomyoma and leiomyosarcoma samples based on Human Genome Organisation Gene Nomenclature Committee and the Catalogue of Somatic Mutations in Cancer

Tumor type	Variant_ id	Samples, n	Sample description	Transcript	Consequence	Human Genome Variation Society		Human Genome Organisation Gene Nomenclature Committee gene identifier	Exons	Catalogue of Somatic Mutations in Cancer identifier
						Coding sequence name	Protein sequence name			
Leiomyoma	chr12-4551244-T-G	4	22LM, 30LM, 32LM, 35LM	ENST00000228837	intron_variant	ENST00000228837.2:c.450+2055A>C	—	FGF6	—	—
	chr11-94192599-G-T	2	16LM, 17LM	ENST00000323929	missense_variant	ENST00000323929.3:c.1475C>A	ENSP00000325863.3:p.Ala492Asp	MRE11A	13/20	—
Leiomyosarcoma	chr10-43597827-C-A	3	LMS12, LMS14, LMS15	ENST00000355710	synonymous_variant	ENST00000355710.3:c.375C>A	ENST00000355710.3:c.375C>A(p.=)	RET	3/20	COSM5021153 COSM5021154
	chr4-81206898-TAA-T	3	LMS04, LMS12, LMS13	ENST00000312465	intron_variant	ENST00000312465.7:c.460-579_460-578delAA	—	FGF5	—	—

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nucleotide polymorphisms. Regarding IMT01, we observed 10 mutations in 8 genes that included approximately 10% of deletions and approximately 90% of single nucleotide polymorphisms (Table S3).

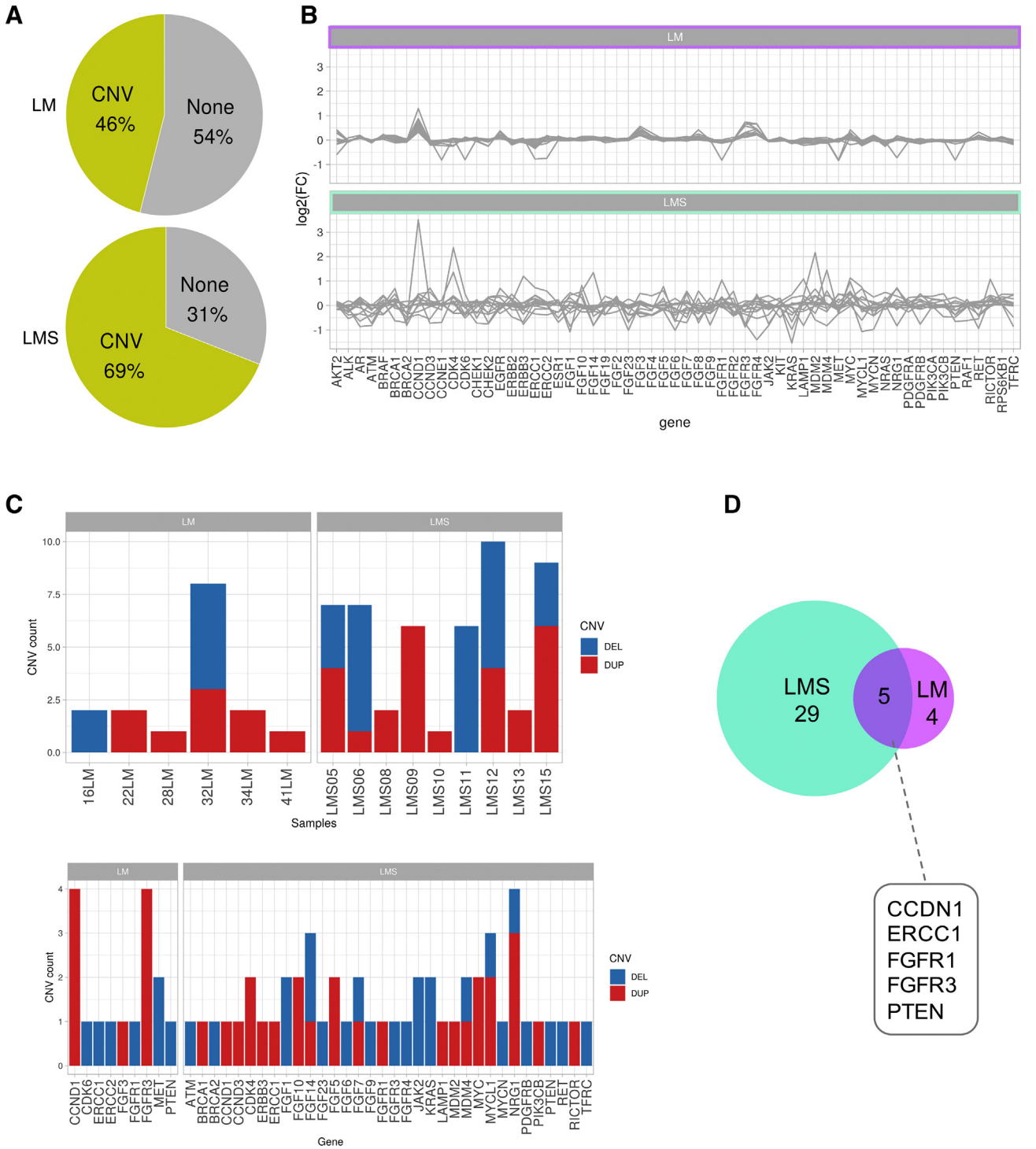
Next, we focused on specific variants in at least 2 leiomyoma or leiomyosarcoma tumors. The most frequently affected variants in leiomyoma were *FGF6* and *MRE11A*, which affected 4 and 2 samples, respectively. In leiomyosarcoma, *RET* and *FGF5* were the most common altered genes, which affected 3 samples (Table 1).

Comparative analysis of CNVs showed that there were more CNVs in leiomyosarcoma (69%) compared with leiomyoma (46%) cases (Figure 1, A; Table S4). Interestingly, when we represented their distribution by specimen group and gene, we observed the highest heterogenicity in leiomyosarcoma, with more deletions and duplications than leiomyoma (Figure 1, B and C). Pairwise comparisons showed significant differences (q-value$\leq$.05).

The most frequent duplications in leiomyoma were on chromosomes 11 and 4, which affect *CCND1* and *FGFR3*; the most frequent deletion was detected on chromosome 7, which affected *MET*. For leiomyosarcoma samples, chromosomes 5, 9, and 12 were the most affected, with deletions encompassing *FGF1, JAK2,* and *KRAS*. The most frequently amplified genes in leiomyosarcoma were *CDK4, FGF10, FGF5,* and *MYC* on chromosome 12, 5, 4, and 8, respectively. Duplications and deletions in the leiomyosarcoma group were in *FGF14, FGF7, MDM4, MYCL1,* and *NRG1* (Figure 1, C; Table 2). For IMT samples, 6 deletions that affect *CCND3, ERBB3, FGF7, JAK2, NRAS,* and *RAF1* and 2 duplications that included *FGF10* and *FGFR4* were found.

Genetic modifications were shared between leiomyoma and leiomyosarcoma in 5 genes: *CCDN1, ERCC1, FGFR1, FGFR3,* and *PTEN*. Although *ERCC1* and *FGFR1* were affected by deletions in leiomyoma, these genes were duplicated in leiomyosarcoma. Conversely, *FGFR3* presented duplications in leiomyoma and deletions in

FIGURE 1
Comparative genomic analysis of leiomyoma and leiomyosarcoma



A, Pie charts show percentage of copy number variations in leiomyoma and leiomyosarcoma (*top to bottom*). **B,** Profile for detected amplifications and deletions in leiomyoma (*top*) and leiomyosarcoma samples (*bottom*) with the use of log2-fold change. **C,** *Barplots* show the distribution of deletions and duplications per sample (*left*). *Barplots* show distribution of deletions and duplications associated by gene (*right*). **D,** Venn diagram represents the number of shared copy number variations by uterine leiomyoma (*right*) and leiomyosarcoma (*left*).

CNV, copy number variation; *FC*, fold change; *LM*, leiomyoma; *LMS*, leiomyosarcoma.

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TABLE 2
Relevant copy number variants in leiomyoma and leiomyosarcoma samples

Tumor type	Genes	Type	Size	Frequency group (%)	Total frequency, %	Supporting studies
Leiomyoma	CCND1	Duplication	13236	30.8	14.8	Musgrove et al (2011)[74]
	FGFR3	Duplication	13328	30.8	14.8	Yu et al (2008)[75]
	MET	Deletion	97040	15.4	7.4	Toro et al (2003)[76]
Leiomyosarcoma	FGF1	Deletion	102108	13.3	7.1	Zhou et al (2016)[77]
	JAK2	Deletion	104804	20	10.7	Hayashi et al (2008)[78]
	KRAS	Deletion	38360	13.3	7.1	Schachtschneider et al (2017)[79]
	CDK4	Duplication	3482	13.3	7.1	Francis et al (2017)[80]
	FGF10	Duplication	83688	20	10.7	Zhou et al (2016)[77]
	FGF5	Duplication	23615	13.3	7.1	Zhan et al (1988)[81]; Giacomini et al (2016)[82]
	MYC	Duplication	4364	13.3	7.1	Jeffers et al (1995)[83]
	FGF14	Deletion/duplication	678848	20	10.7	Presta et al (2017)[84]
	FGF7	Deletion/duplication	40556	20	10.7	Zhou et al (2016)[77]
	MDM4	Deletion/duplication	39564	13.3	7.1	Toledo and Wahl (2007)[85]; Atwal et al (2009)[86]
	MYCL1	Deletion/duplication	4517	20	10.7	Barnabas et al (2014)[87]; Groisberg et al (2017)[88]
	NRG1	Deletion/duplication	1124420	26.7	14.3	Yatsenko et al (2017)[57]

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leiomyosarcoma; no differences between leiomyoma and leiomyosarcoma were detected for *CCND1* and *PTEN*. Finally, 4 CNVs were found in leiomyoma; 29 CNVs were present exclusively in leiomyosarcoma (Figure 1, D).

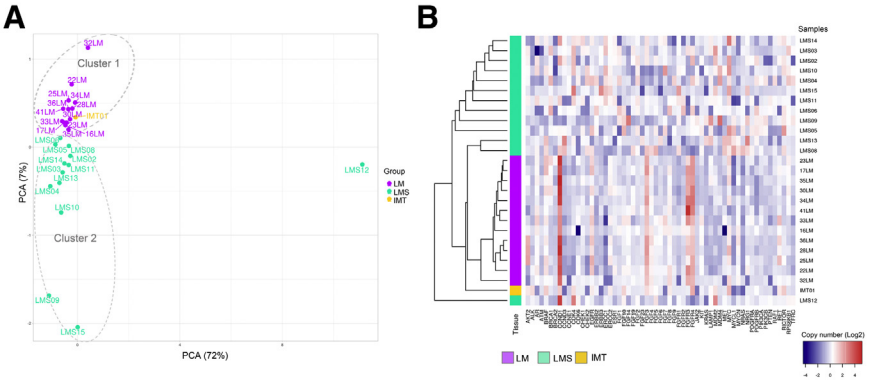
Principal component analysis demonstrated that leiomyoma and leiomyosarcoma samples clustered separately according to tissue of origin except for LMS12, which was considered an outlier (Figure 2, A). Interestingly, IMT01, initially diagnosed as leiomyosarcoma, was grouped among leiomyoma samples, which suggests an additional molecular subtype. Unsupervised hierarchical clustering analysis recreated the principal component analysis clustering structure (Figure 2, B). As previously observed, leiomyoma specimens were grouped in a homogeneous cluster that encompassed 13 samples, although leiomyosarcoma samples were more heterogeneous. Specifically, we observed 1 main cluster that included 10 leiomyosarcoma samples; another 2 samples (LMS08 and LMS13) clustered separately, and LMS12 which was considered an outlier characterized by distinctive alterations in *CCDN1* (Figure 2, B). To note, the IMT sample showed specific alterations in *AKT2, ALK,* and *FGF7* clustered separately from leiomyoma and leiomyosarcoma groups, which supported a different molecular subtype.

Differentially expressed genes in leiomyoma and leiomyosarcoma

Transcriptome sequencing results identified 3 groups: a homogeneous group with leiomyosarcoma samples (cluster 1), a homogeneous group composed by leiomyoma (cluster 2), and a heterogeneous group composed by leiomyoma, leiomyosarcoma, and IMT samples (cluster 3; Figure 3, A). Unsupervised hierarchical clustering also categorized 3 expression clusters. In cluster 1, leiomyosarcoma samples were together into the same group; cluster 2 corresponded with a homogeneous group that included leiomyoma samples, and cluster 3 included some of leiomyosarcoma samples, the IMT specimen, and 2 leiomyoma samples (17LM and 25LM), which supported our previous results (Figure 3, B).

Next, we identified targetable differential expression in leiomyosarcoma and leiomyoma. Overall, 11 of 55 genes (*ALK, BRCA2, FGFR3, FGFR4, FLT3, NTRK1, PAX3, PAX7, RET, ROS1,* and *TMPRSS2*) were up-regulated significantly in leiomyosarcoma compared with leiomyoma ($P \leq .5$; Figure 3, C; Table S5). These differentially expressed genes were then evaluated for molecular functions and biologic processes, considering only pathways with at least 2 annotated genes. The Kyoto Encyclopedia of Genes and Genomes database analysis of implicated functions revealed an overrepresentation of pathways that were involved in transcriptional misregulation and central carbon metabolism in cancer as well as RAS/MAPK and PI3K-AKT signaling pathways and

FIGURE 2
Clustering of leiomyoma, leiomyosarcoma, and inflammatory myofibroblastic tumor samples based on copy number variation



A, Principal component analysis of leiomyoma (n=13), leiomyosarcoma (n=13), and inflammatory myofibroblastic tumor (n=1) samples. Each sample is represented in the Figure as a colored point (*green*, leiomyosarcoma; *purple*, leiomyoma; *yellow*, inflammatory myofibroblastic tumor). Most variance between both groups is captured in the first 2 principal components. **B,** Heatmap dendrogram of copy number variations associated with genes (*column*) and for each analyzed sample (*row*) of leiomyoma (*purple*), leiomyosarcoma (*green*), and inflammatory myofibroblastic tumor (*yellow*). Copy number profiles include frequent amplifications (*red*) and deletions (*blue*). Horizontal length of each arm reflects relatedness of clusters.

IMT, inflammatory myofibroblastic tumor; *LM*, leiomyoma; *LMS*, leiomyosarcoma; *PCA*, principal component analysis.

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thyroid cancer ($P \leq .05$; Figure S1, A). Moreover, gene ontology enrichment analysis showed protein tyrosine kinase activity as a main molecular function involved in the tumorigenic process (Figure S1, B) and peptidyl-tyrosine modification/phosphorylation as a principal biologic process (Figure S1, C).

Novel *ALK Receptor Tyrosine Kinase-Tensin 1* fusion

Although RNA-seq was performed with the use of paired-end sequencing, we could detect fusion transcripts from 55 genes targeted by the TST170 panel, which met a minimum threshold score of ≥ 0.98. One sample, IMT01, which initially was diagnosed as LMS01, showed an *ALK Receptor Tyrosine Kinase — Tensin 1 (TNS1)* fusion (Figure 4, A). We used immunohistochemistry and FISH to validate the *ALK* rearrangement.[40,41] As shown, immunohistochemistry (Figure 4, B) demonstrated diffused strong ALK-positive staining that was confirmed by FISH to be an *ALK* translocation (Figure 4, C).

Overview of data for specific pathways and targetable mutations

Gene expression data revealed 5 genes (*FGFR4, PAX3, PAX7, ROS1, and TMPRSS2*) with detected mutations in at least 10 tumors and 18 genes of 23 (78%) from CNVs, small variants, and gene expression were mutated in at least 2 tumors (Figure 5). Interestingly, *PAX3* was the most frequent mutated gene that resulted in messenger RNA up-regulation, although *NRG1* was also altered at CNV level. Overall, although LMS02 and LMS04 were less altered, 85% of tumors were affected with at least 11 mutations, which indicated the complexity of the tumorigenic process (Figure 5).

The Kyoto Encyclopedia of Genes and Genomes database identified 20 pathways, mainly related to cancer and cell cycle; the PI3K/AKT pathway was the most representative (Figure S2, A). We identified main up-regulated genes from the integrative analysis and interactions with other represented pathways, such as RAS/RAP1 signaling pathway, MAPK, and p53 (Figure S2, B).

Implicated molecular functions (Figure S2, C and D) and biologic processes (Figure S2, E and F) for leiomyosarcoma established a relationship network with interesting connections. The molecular function network highlighted 5 significant categories: (1) protein tyrosine kinase activity, (2) ras guanyl-nucleotide exchange factor activity, (3) transmembrane receptor protein tyrosine kinase activity, (4) phosphatidylinositol biphosphate 3-kinase activity, and (5) phosphatidylinositol-4-5-biphosphate 3-kinase activity (Figure S2, C). All functions were connected by integrated genes that belonged to >1 function. Specifically, genes from the *FGF* family were shared by all functions (Figure S2, D). Regarding biologic processes, peptidyl-tyrosine modification, phosphorylation, and inositol lipid/phosphate-mediated signaling were the most representative processes (Figure S2, E), which were regulated by *ALK, FLT3, ROS1, RET, NTRK1, JAK2,* and *FGF* family genes, the latter with more shared functions than observed for molecular function (Figure S2, F).

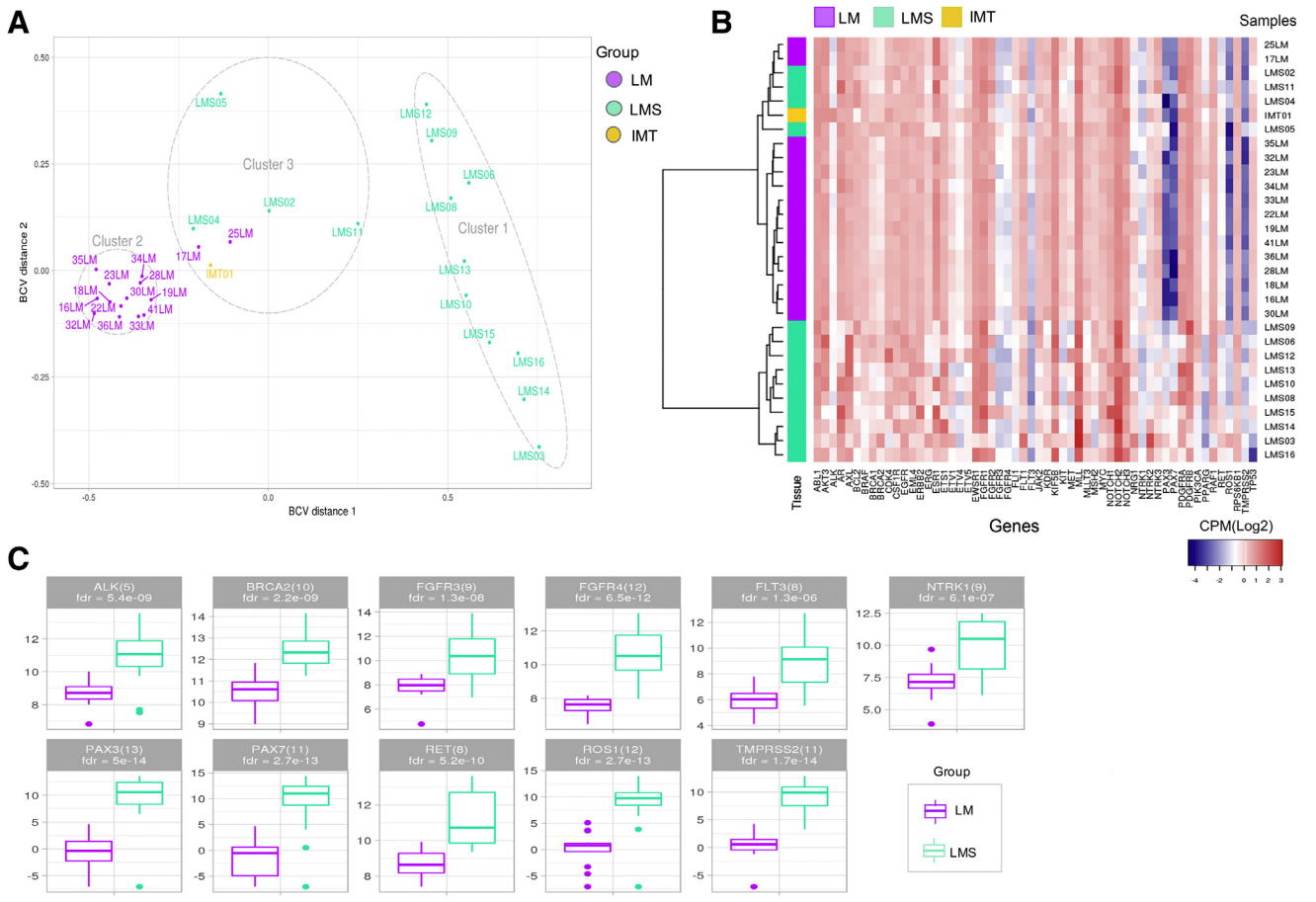
Comment

Principal findings

The present study represents a scientific-technologic innovation that allows, with the use of the genomic tools, to shift the current diagnostic focus. By using next generation sequencing, we have been able to identify genetic variants and potential genomic markers in leiomyoma, leiomyosarcoma, and IMT samples. In our hands, a combination of genomic and transcriptomic findings allowed us to differentiate among all these myometrial tumors.

Based on our findings, we propose that future studies to differentiate among uterine leiomyosarcoma and leiomyoma should be guided by next generation sequencing because it could address some biologic issues (discordant results among immunohistochemistry and FISH techniques because of subjective signal interpretation) by detecting mutations/amplifications or posttranslational changes through an objective approach.

FIGURE 3
Targeted transcriptional profile for the 55 genes included in the TruSeq Tumor 170 gene panel



A, Multidimensional scaling plot of distances in leiomyoma (n=13), leiomyosarcoma (n=13), and inflammatory myofibroblastic tumor (n=1) samples in gene expression profiles. Each sample is represented in the Figure as a colored point (*green*, leiomyosarcoma; *purple*, leiomyoma; *yellow*, inflammatory myofibroblastic tumor). Most variance between both groups is captured in the first 2 principal components. **B,** Heatmap dendrogram of expression of the 55 genes analyzed (*column*) for each sample (*row*) shows 3 clusters of samples. **C,** Boxplot for 11 genes significantly up-regulated in leiomyosarcoma (*green*) vs leiomyoma (*purple*). The false discovery rate value is represented for each gene. Numbers between parenthesis refer to the number of altered samples.

IMT, inflammatory myofibroblastic tumor; *LM*, leiomyoma; *LMS*, leiomyosarcoma.

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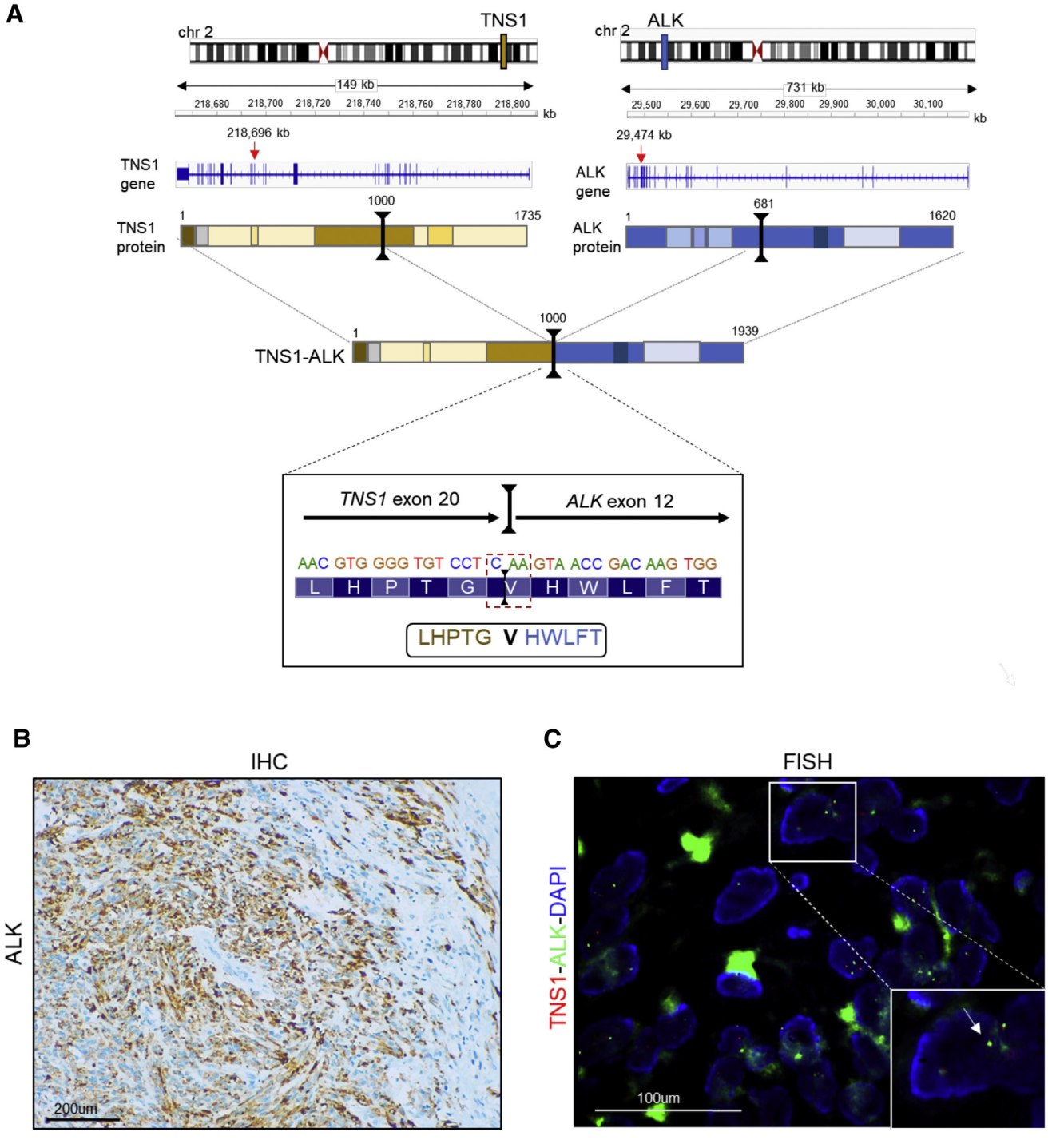
Results in context

Numerous genes can influence tumor progression via several types of variations.[42–48] Our findings indicate that leiomyosarcomas are more unstable, with a higher incidence and heterogenicity than leiomyoma. Specifically, most cases that were analyzed for CNVs demonstrated more losses than gains, being also present in some chromosomal regions that contain fibroblast growth gene (FGF1), protooncogenes like *KRAS* and nonreceptor tyrosine kinase genes such as *JAK2*. Additionally, we found 29 exclusive affected genes in leiomyosarcoma, although only 4 were present in leiomyoma.

Principal component analysis allowed us to gain an overview of the data by showing that samples with the same tumor type clustered together with only 2 outliers: LMS12 and IMT01 (initially diagnosed as LMS01; Figure 2, A). These results were then confirmed with an associated dendrogram that was divided into 2 main branches: 1 branch contained tight clusters of leiomyoma (cluster 1) and another branch with a majority of leiomyosarcoma (cluster 2). To note, LMS08 and LMS13 had an intermediate pattern between leiomyoma and leiomyosarcoma, which suggests an additional molecular subtype. However, because they were obtained commercially, we have some limitations to validate our results and to obtain their clinical profile. Conversely, we could confirm that IMT01 represented an additional molecular subtype.

At the transcriptomic level, we identified 3 groups: a homogeneous group with leiomyosarcoma samples (cluster

FIGURE 4
Detection of a novel ALK-TNS1 fusion transcript in inflammatory myofibroblastic tumor specimen that was initially diagnosed as leiomyosarcoma



A, Schematic representation of the gene sequence and main functional domains of proteins for *TNS1* and *ALK*. In the gene sequence, the *red arrow* indicates the exon where the fusion was detected. In the protein scheme; *black lines* represent breakpoints, and *dashed lines* indicate a closer view of the transcript fusion point. Amino acid sequence at the fusion point is highlighted in the *rectangle*. **B,** Immunohistochemistry staining of intense cytoplasmic staining for ALK in the IMT01 sample. Scale bar represents 200 μM. **C,** Representative image of fluorescence in situ hybridization for *ALK* shows several nuclei harboring split and fused signals (*arrows*).

FISH, fluorescence in situ hybridization; *IHC*, immunohistochemistry.

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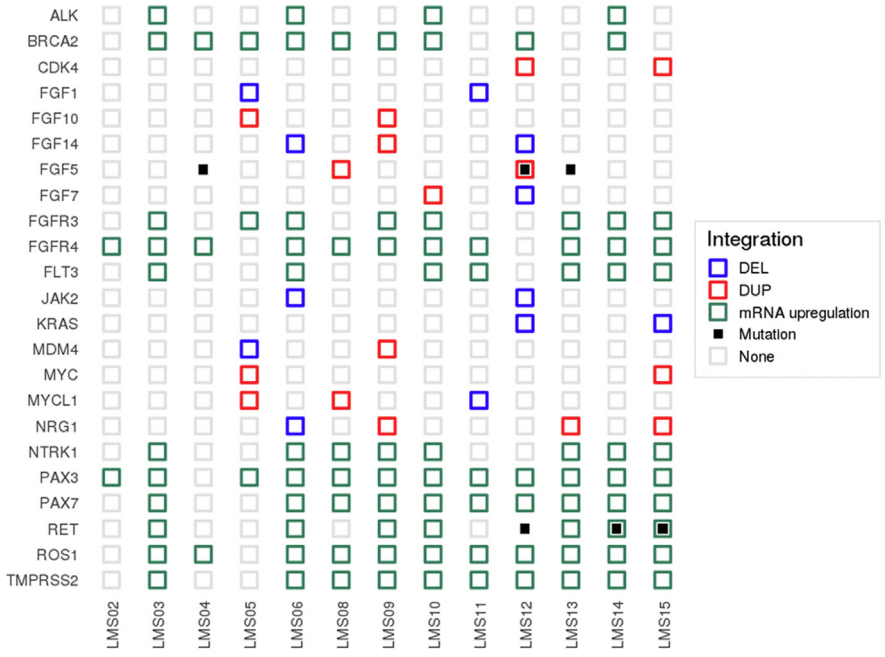
FIGURE 5
Representation of recurrently affected genes in leiomyosarcomas



The columns represent samples, as indicated at the bottom; most representative genes are shown by rows. *Grey boxes* indicate unaffected genes; *blue boxes* indicate genes affected with deletions, and *red boxes* denote duplications. Mutations are represented by *black squares*; highlighted *green squares* indicate messenger RNA up-regulation.

DEL, deletions; *DUP*, duplications; *mRNA*, messenger RNA.

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1), a homogeneous group composed by leiomyoma (cluster 2), and a heterogeneous group composed by leiomyoma, leiomyosarcoma, and IMT specimens (cluster 3), which were confirmed by hierarchic clustering and gene set enrichment. Additionally, differential expression of *PAX3, PAX7, ROS1,* and *TMPRSS2* may contribute to classification of the outliers. Nevertheless, because of some technical limitations of the methods used, additional efforts, in terms of global RNAseq approaches and higher number of samples analyzed, should be required for further clarification in samples with an intermediate profile (Figure 3, A: cluster 3). In that sense, gene ontology and gene set enrichment analysis provide structured functional and biologic process information about these individual genes, because pathways that are involved in transcriptional misregulation and central carbon metabolism in cancer were overrepresented. Additionally, we also identified key pathways of RAS/MAPK and PI3K-AKT, which play important roles in cancer-related processes, such as cell growth, survival, and apoptosis. These results agree with earlier published studies.[49]

Furthermore, we identified a novel *TNS1—ALK* fusion in IMT01 sample, initially diagnosed as leiomyosarcoma. *TNS1* encodes tensin 1, which crosslinks actin filaments and acts as an oncogenic driver in chromosomally unstable colorectal cancer.[33,50] *ALK* is found frequently in fusions in patients with non—small cell lung cancer[51—53] and in IMTs of the female genital tract.[54] Because these are under-recognized smooth muscle tumors, the distinction of IMT, leiomyoma, and leiomyosarcoma can be subtle.[55] In fact, IMT01 sample, which previously was diagnosed as leiomyosarcoma, was instead established as IMT when ALK staining was detected by immunohistochemistry and confirmed the translocation by FISH.

Our integrated analysis also revealed numerous potential target genes like *FGFR4, PAX3, PAX7, ROS1,* and *TMPRSS2* with detected mutations in at least 10 tumors. Among them, *PAX3* was the most frequent mutated gene that resulted in messenger RNA up-regulation, although *NRG1* was also altered at CNV level. Interestingly, it has been reported that dysregulation of PAX family members contributes to tumorigenesis in soft tissue sarcomas by altering signaling pathways that affect proliferation, cell death, myogenic differentiation, and migration.[56] Regarding *NRG1,* there is evidence that it acts as a tumor suppressor gene and that its dysregulation has been linked to tumorigenesis.[57,58]

To better understand the tumorigenic process, networks between functions and genes highlighted protein tyrosine kinase activity and peptidyl-tyrosine phosphorylation as the main categories for molecular functions and biologic process, respectively. Interestingly, both receptor and nonreceptor tyrosine kinases have emerged as clinically useful drug target molecules for the treatment of certain types of cancer, being leiomyosarcoma highly expressed tyrosine kinase another potential drug targetable cancer.

Clinical implications

The main problem in diagnosing leiomyoma and leiomyosarcoma is the absence of risk factors and standardized criteria to identify them before surgery as benign or malignant, because currently there are no molecular biomarkers used in clinical practice. This situation could be the origin of significant stress in the patient, leading to unnecessary invasive procedures and additional costs to the National System of Health.

Nowadays, the application of next generation sequencing enables the detection of new mutations that, when coupled to bioinformatic tools, advances our understanding of chromosomal/genetic instability.

Detection of cell-free DNA is now a reality as a biomarker for the detection of tumor DNA mutations in peripheral

blood, urine, or other fluids as personalized therapy in cancer diagnosis and tumor progression[59]; the gynecologic cancers should be no exception. Future studies will explore the translational application of this panel in circulating cell-free tumor DNA.

Research implications

Some reports have described the use of next generation sequencing in leiomyoma and leiomyosarcoma,[27–29,32] which suggests that differing mechanisms underlie these tumorigenic mutations. In this sense, our study demonstrates consistent genetic differences between leiomyoma and leiomyosarcoma.

At RNA level, recent studies have highlighted the importance of gene fusions and splice variants in solid tumors, because a single chimeric RNA transcript could result from numerous DNA alterations.[60,61] We identified a novel *TNS1—ALK* fusion in 1 IMT sample, previously diagnosed as leiomyosarcoma. Fortunately, IMT has a less aggressive clinical course compared with most metastatic leiomyosarcoma, as was the case of the patient analyzed in our study, who remained alive with disease after 2 years (Table S2). In this sense, molecular diagnosis could overcome the limitations of conventional analyses.

Finally, we have been able to identify pathways that are affected differentially in leiomyoma vs leiomyosarcoma. In fact, the comparison of our results with previously published studies reinforces the importance of certain specific pathways such as RAS/RAP1 signaling pathway, MAPK, and p53.[62,63] For instance, the PI3K/AKT/mTOR pathway is activated in approximately 30—40% of breast cancer cases. In triple-negative breast cancer, oncogenic activation of the PI3K/AKT/mTOR pathway can happen as a function of overexpression of upstream regulators such as EGFR, activating mutations of *PIK3CA*, loss of function or expression of PTEN, and the proline-rich inositol polyphosphatase, which are down-regulators of PI3K. This is consistent with the hypothesis that PI3K inhibitors can overcome resistance to endocrine therapy.

Strengths and limitations

Although promising, our results are limited to the 170-solid tumor-associated genes that we investigated. Further, because of the rarity and limited availability of leiomyosarcoma samples, our study consisted of a small sample of 13 leiomyosarcoma cases that does not allow for large-scale genomic analysis. Therefore, additional studies with larger numbers of samples and clinical outcomes are needed.

Conclusion

In conclusion, this study provides promise in differentiation of leiomyoma and leiomyosarcoma at the molecular level, which increases our understanding of the genetic basis for these tumors. By using high throughput sequencing, we have been able to identify novel genes and pathways that are affected differentially in leiomyosarcoma vs leiomyoma. The ones with more applicability and potential as diagnostic markers are ALK, BRCA2, CDK4, FGF1, FGF10, FGF14, FGF5, FGFR3, FGFR4, FLT3, JAK2, KRAS, MDM4, MYC, MYCL1, NRG1, MTRK1, PAX3, PAX7, RET, ROS1, TMPRSS2, mainly related with the PI3K/AKT, RAS/RAP1, MAPK, and p53 signaling pathways. ■

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Supplemental Information for Materials and Methods

Study participants and sample collection

Participants in this study were 18−62-year-old women who had undergone hysterectomy or laparoscopic/laparotomic myomectomy as surgical treatment for symptomatic leiomyoma and/or suspected leiomyosarcoma at the Department of Obstetrics and Gynecology, University Hospital La Fe in Valencia, Spain. Patients with other gynecologic disorders, malignancies, or diagnosed bacterial, fungal, or viral infections were excluded. Use of human tissue specimens from these patients was approved by the Institutional Review Board of University Hospital La Fe (2016/0118). All patients provided written informed consent before inclusion in the study. Clinical data were obtained for these cases, after which samples were anonymized for the study.

A total of 27 formalin-fixed paraffin-embedded (FFPE) tumor samples were included in this study; 18 of them were collected from Hospital La Fe, including leiomyoma (n=13), inflamatory myofibroblastic tumor initially diagnosed as leiomyosarcoma (n=1) and leiomyosarcoma (n=4). Nine leiomyosarcoma samples were provided by Origene Technologies Inc (Rockville, MD; Table S1). All tumors were evaluated by 2 expert pathologists and histologically confirmed according to World Health Organization criteria.[35,36] Leiomyosarcoma samples were also assessed for mitotic activity, presence/absence of necrosis, and other pathology annotations (Table S2).

Nucleic acid extraction

We extracted nucleic acids from 5 μm-thick FFPE tumor samples using GeneRead DNA kit and miRNeasy FFPE kit (Qiagen GmbH, Hilden, Germany), per manufacturer's instructions. Genomic DNA (gDNA) and RNA samples were quantified by spectrophotometric (Nanodrop 2000; Thermo Scientific, Waltham, MA) and fluorometric (Qubit dsDNA BR Assay Kit; Thermo Scientific) methods. DNA integrity profile was assessed with the High Sensitivity DNA Assay (Agilent, Santa Clara, CA). Additionally, we measured amplification potential of nucleic acids by assessing ΔCq value using quantitative polymerase chain reaction after normalization to a fixed input mass.

RNA integrity profile was measured with the Standard Sensitivity RNA kit (Advanced Analytical Technologies, Ankeny, IA); a fragment analyzer was used to determine DV200 value, which measures the percentage of RNA fragments >200 nucleotides in length. We assessed quality control of samples using an FFPE QC kit (Illumina, San Diego, CA). Quality control metrics ensured accurate variant calling, with ≥95% sensitivity and specificity. Only DNA samples with ΔCq ≤5 and a concentration of 3.5−10 ng/μL and RNA samples with DV200 ≥20% and a concentration of ≥4.5 ng/μL were included in this study.

Targeted sequencing library preparation

DNA and RNA libraries were sequenced simultaneously with the Illumina TruSight Tumor 170 kit (Illumina, San Diego, CA), which covers the coding regions of 170 genes that are associated with solid tumors. Complementary DNA was synthesized from RNA samples, and gDNA samples were fragmented to 90−250 base pair with the use of a S220 focused ultrasonicator (Covaris, Woburn, MA). Sheared gDNA and complementary DNA were converted into sequenceable libraries.

Regions of interest were hybridized to biotinylated probes, magnetically pulled down with streptavidin-coated beads, and eluted to enrich the library pool. Libraries were normalized with the use of a simple bead-based protocol before being pooled and sequenced. Paired-end read sequencing was performed with the use of an Illumina NextSeq 500. Sequence data were analyzed via BaseSpace App TruSight Tumor 170 (version 1.0; Illumina), which provides call files for copy number variants (CNV) and small variants from DNA libraries. Along with a DNA workflow, the assay also included an RNA workflow to identify splice variants and gene fusions.

DNA analysis: small variants and CNVs

DNA sequence data were demultiplexed (Illumina FASTQ 1.9 files with Phred+33; Wellcome Sanger Institute, Cambridge, UK) to generate intermediate analysis files in FASTQ format, which contain at least 65 million DNA reads for each sample. Briefly, sequence data were mapped to the human hg19 genome (GRCh37) with the use of Isaac alignment software 3.16.02.19.

Small variants, which include point mutations and indels, were detected by Pisces (Illumina),[37] and CNVs were detected by CRAFT (https://goo.gl/ZHruxH). The CRAFT CNV caller performed amplification, reference, and deletion calling for target amplification genes within the assay, based on calculated fold-change value. Pisces performed somatic variant calling to identify variants at low frequency in DNA samples.[37] Variants with an allele frequency >1% in ExAC Browser or >1% in gnomAD Browser[64] (http://gnomad.broadinstitute.org/) were filtered out. We retained variants with variant fraction >0.2 and coverage >6.

RNA analysis

Gene expression

Differential expression analysis was performed using the edgeR package[38] from Bioconductor software.[39] Trimmed mean of M-values technique was used to compute factors of normalization. Tagwise dispersions were calculated and subjected to an exact test.[38] Benjamini−Hochberg multiple testing correction was applied. ClusterProfiler package[65] from Bioconductor software was used to analyze pathways of significant differentially expressed RNAs. Annotated genes that express linear RNA were matched to their respective gene ontology terms, and an over-representation test was performed.

Fusions and splice variants

RNA sequence data were demultiplexed to generate intermediate analysis FASTQ format files that contained at least 20 million RNA reads per sample. STAR Alignment software was used to align reads to the human hg19 genome

(GRCh37) and gene transcripts. Fusion genes were identified with Manta RNA Fusion Calling software (Illumina), and RNA Fusion Variant Filtering software (Sunquest, Tucson, AZ) was used to deliberate filters and reduce false-positive calling from sequence homologs, polymerase read-through, or FFPE artifacts. RNA Splice Variant Caller software (Illumina) was used for splice variant calling; the Illumina Annotation Engine annotated splice variants.

Gene fusion validation

To validate the rearrangement and uncommon ALK Receptor Tyrosine Kinase fusion partner, we performed immunohistochemistry and fluorescence in situ hybridization (FISH). For ALK immunohistochemistry, 4-μm FFPE tissue sections were assessed with the use of a 1:10 dilution of the mouse monoclonal antibody 5A4 (Roche Diagnostics, Mannheim, Germany). Negative controls were produced by eliminating the primary antibody from the diluents. A Leica Bond III automated staining platform (Leica Biosystems, Mount Waverley, Victoria, Australia) was used with heat-induced epitope retrieval, per manufacturer's instructions. Percentage of labeled tumor cells and intensity of cytoplasmic or nuclear ALK staining were assessed independently by 2 pathologists.

For FISH, 2-μm FFPE tissue sections were assessed with the use of a Vysis ALK Break Apart Probe kit (Dako, Agilent Technologies, Santa Clara, CA), according to manufacturer's recommendations. In brief, slides were pretreated at 95°C and then digested with pepsin for 2 minutes at 37°C with the use of the histology FISH accessory Kit (Dako, Glostrup, Denmark). Next, they were denatured for 3 minutes at 73°C and incubated for 16 hours at 37°C with ALK probes (1:10 dilution). Finally, slides were washed, dehydrated before counterstaining, and analyzed with a Nikon Eclipse 80i fluorescence microscope (Amsterdam, Netherlands). A minimum of 100 nuclei were scored and interpreted by 2 pathologists who used standard criteria.[66] Cases were considered positive for *ALK* rearrangement when 2 signal diameters were seen between red and green signals in >30% of tumor cells.

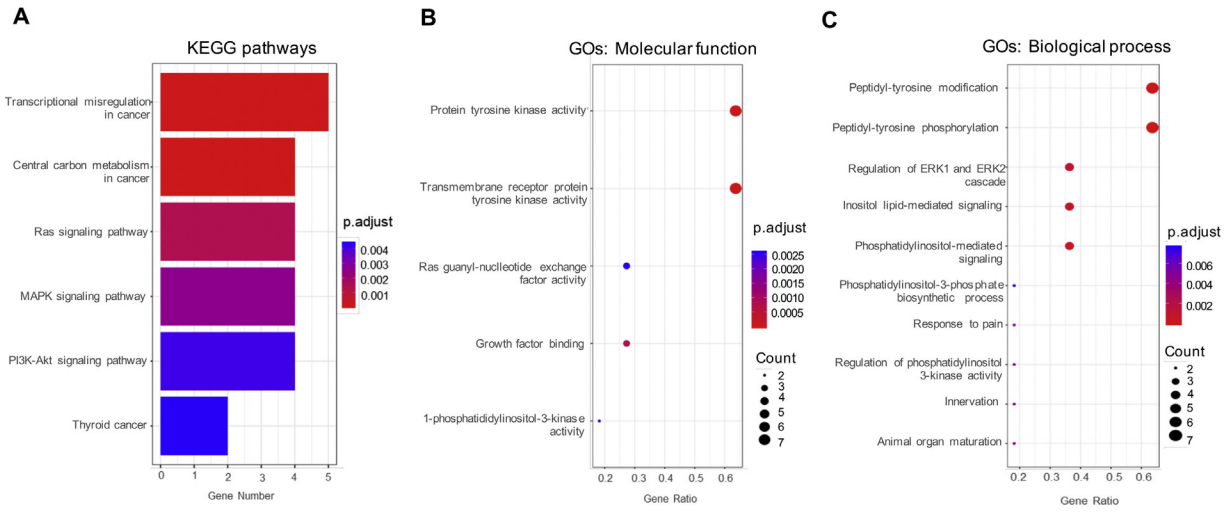
Integration of data and pathway analysis

Small variants, CNVs, differential expression, and fusions were integrated to generate a combined output. Additionally, Kyoto Encyclopedia of Genes and Genomes, gene ontology enrichment, and network analysis were performed with ClusterProfiler. Regarding the background/universe gene list, 7471 genes were annotated by Kyoto Encyclopedia of Genes and Genomes. Additionally, 17,976 annotated genes were related with molecular function ontology, and 18,466 annotated genes were related with biologic process ontology. Correction for multiple testing was performed with the use of the false discovery rate.[67]

Statistical analysis

Statistical analysis was performed using R version 3.3.2 (http://www.R-project.org). Specifically, to perform the hierarchic clustering, the manhattan distance and ward.D2 agglomeration method was used for CNV; manhattan distance and ward.D agglomeration method was used for RNA. Additionally, gene expression was performed with EdgeR package. Ggplot2,[68] tidyverse,[69] and VennDiagram[70] packages from CRAN and VariantAnnotation,[71] edgeR, and heatmap3[72] from Bioconductor were used.[73]

FIGURE S1
Functional meaning of transcriptomic signature for the tumorigenic process



A, Distribution of implicated functions based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, where pathways are represented on the *y-axis* and the number of genes that belong to each pathway are detailed on the *x-axis*. **B,** Gene ontology enrichment analysis of molecular functions that contain pathway name and gene ratio from the annotated signature. **C,** Gene ontology enrichment analysis of biologic process. The probability-adjust value representation was shown as a *gradient color from blue to red*.

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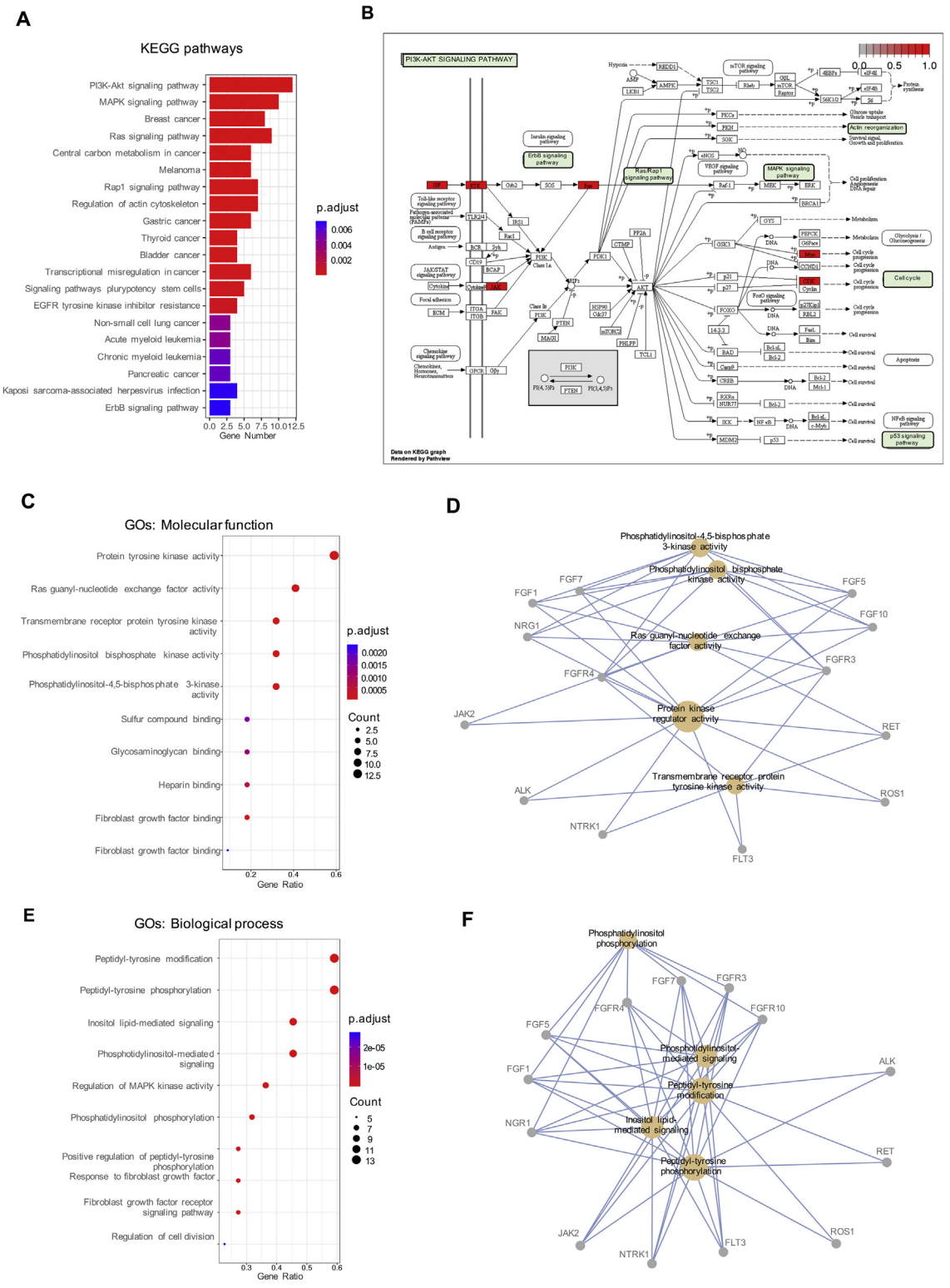
FIGURE S2
Functional meaning of integrated signature for the tumorigenic process



A, Distribution of implicated functions based on the Kyoto Encyclopedia of Genes and Genomes pathway database, where pathways that are classified based on Benjamini and Hochberg probability-adjust value are represented on the *y-axis* and the number of genes belonging to each pathway are detailed on the *x-axis*. **B,** PI3K-AKT signaling pathway diagram that contains fold-change representation for most integrated genes that belong to this pathway. **C,** Functional gene annotation in gene ontology for specific molecular functions based on probability-adjust value. **D,** Network modeling of gene expression and functional relationship between all specific processes that are related to molecular functions. *Big nodes* represent main categoric functions in the related process; *small nodes* represent genes obtained by integration analysis. **E,** Functional gene annotation in gene ontology for specific biologic processes based on probability-adjust value. **F,** Network modeling of gene expression and functional relationship between all specific biologic processes.

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TABLE S1
Clinical and pathologic features of patients who were diagnosed with leiomyoma and leiomyosarcoma

Tumor type	Case identification	Source	Age	Ethnicity	Parity	Miscarriage	Clinical history	Surgical procedure	Tumor size, mm	FIGO staging classification
Leiomyoma	16LM	La Fe[a]	39	Caucasian	No	No	N/A	Laparotomic myomectomy	60	4
	17LM	La Fe	47	Caucasian	Yes	Yes	Sterility	Laparotomic myomectomy	65	5
	22LM	La Fe	47	Caucasian	Yes	No	Family history	Laparoscopic hysterectomy	68	4
	23LM	La Fe	47	Caucasian	Yes	Yes	Family history	Laparoscopic hysterectomy	60	5
	25LM	La Fe	47	Caucasian	Yes	Yes	Family history	Laparoscopic hysterectomy	110	6
	28LM	La Fe	39	Caucasian	No	No	Family history	Laparotomic myomectomy	82	7
	30LM	La Fe	46	Caucasian	Yes	Yes	Family history	Laparoscopic hysterectomy	86	5
	32LM	La Fe	45	Caucasian	Yes	Yes	N/A	Laparoscopic hysterectomy	12	4
	33LM	La Fe	30	Caucasian	No	No	N/A	Laparotomic myomectomy	90	2-5
	34LM	La Fe	40	Caucasian	No	No	N/A	Laparotomic hysterectomy	150	8
	35LM	La Fe	48	Caucasian	Yes	Yes	Family history	Laparotomic hysterectomy	35	4
	36LM	La Fe	48	Caucasian	Yes	No	N/A	Laparoscopic hysterectomy	70	4
	41LM	La Fe	44	Caucasian	Yes	No	N/A	Laparoscopic hysterectomy	24	2-5
Leiomyosarcoma	LMS02	La Fe	50	Latin	Yes	Yes	N/A	Laparotomic hysterectomy	90	IB
	LMS03	La Fe	64	Caucasian	N/A	N/A	N/A	Laparotomic hysterectomy	230	IV
	LMS04	La Fe	53	Caucasian	Yes	No	N/A	Laparotomic hysterectomy	120	IV
	LMS05	La Fe	55	Caucasian	Yes	No	Family history	Laparotomic hysterectomy	200	IIIB
	LMS06	Origene[b]	39	Asian	N/A	N/A	N/A	N/A	N/A	N/A
	LMS08	Origene	50	Caucasian	N/A	N/A	N/A	N/A	N/A	IIB
	LMS09	Origene	54	Caucasian	N/A	N/A	N/A	N/A	N/A	IC
	LMS10	Origene	55	Caucasian	N/A	N/A	N/A	N/A	N/A	IIB
	LMS11	Origene	55	Caucasian	N/A	N/A	N/A	N/A	N/A	IC
	LMS12	Origene	56	Caucasian	N/A	N/A	N/A	N/A	N/A	IIIB
	LMS13	Origene	60	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	LMS14	Origene	62	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	LMS15	Origene	67	Caucasian	N/A	N/A	N/A	N/A	N/A	IIIA

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(continued)

TABLE S1
Clinical and pathologic features of patients who were diagnosed with leiomyoma and leiomyosarcoma *(continued)*

Tumor type	Case identification	Source	Age	Ethnicity	Parity	Miscarriage	Clinical history	Surgical procedure	Tumor size, mm	FIGO staging classification
Inflamatory myofibroblastic tumor	IMT01[a,c]	La Fe	60	Caucasian	Yes	No	N/A	Laparoscopic hysterectomy	80	IB

IMT, imflamatory myofibroblastic tumor; *LM*, leiomyoma; *LMS*, leiomyosarcoma; *N/A*, not applicable.

[a] Department of Obstetrics and Gynecology, University Hospital La Fe Valencia, Spain; [b] Origene Technologies Inc, Rockville, MD; [c] Initially diagnosed as leiomyoma and subsequently confirmed as inflamatory myofibroblastic tumor.

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TABLE S2
Morphologic and histologic characteristics of leiomyosarcoma tumors and patient follow up

Case identification	Tumor differentiation	Necrosis	Mitotic activity, high-power field	Atypia	Hystologic variant	Outcome follow up
IMT01[a]	N/A	Present	>19/10	Moderate	N/A	Alive
LMS02	Poorly differentiated	Present	>19/10	Severe	Myxoid	Alive
LMS03	Moderated differentiated	Present	1–9/10	Moderate	Spindle cell	Deceased
LMS04	Poorly differentiated	Present	>19/10	Moderate	Spindle cell	Deceased
LMS05	Poorly differentiated	Present	>19/10	Severe	Spindle cell	Deceased
LMS06	Well differentiated	Absent	>10/10	Moderate/severe	Spindle cell	N/A
LMS08	N/A	Absent	N/A	N/A	N/A	N/A
LMS09	Poorly differentiated	Absent	>40/10	Moderate	N/A	N/A
LMS10	Poorly differentiated	Present	>20/10	Moderate/severe	Spindle cell	N/A
LMS11	Well differentiated	Absent	<10/10	Moderate	N/A	Alive
LMS12	N/A	Absent	N/A	N/A	N/A	N/A
LMS13	Well differentiated	Present	>10/10	N/A	N/A	N/A
LMS14	N/A	Present	N/A	N/A	N/A	N/A
LMS15	N/A	Present	N/A	N/A	Myxoid	N/A

IMT, inflammatory myofibroblastic tumor; *LMS*, leiomyosarcoma; *N/A*, not applicable.

[a] Initially diagnosed as leiomyosarcoma and subsequently confirmed as inflamatory myofibroblastic tumor.

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TABLE S3
Affected genes and actionable mutations in leiomyoma and leiomyosarcoma groups

Tumor type	Mean variants per sample, n	Genes, n	Gene description	Samples (n)	Deletions, n (%)	Insertions, n (%)	Single nucleotide polymorphism, n (%)
Leiomyoma	20	82	*FGFR2,KLLN,PTEN,ATM,KMT2A,MTOR,NRAS,NOTCH2, FGF19,AP001888.1,FGF3,MRE11A,MDM4,PTPN11, SDCCAG8,FGF6,ERBB3,MDM2,NA,LAMP1, FGF9, FLT1,BRCA2,MYCL,RP11-982M15.2,MPL,HPDL, SLC35F4,RAD51B,RAD51,IDH2,TSC2,SLX4,CREBBP, RAD51L3-RFFL,TP53,RBFOX3,STK11,NOTCH3, TGFBR3,AKT2,GNAS-AS1,ERG,MYCNOS,BARD1, EP300,DNMT3A,MSH2,MSH6,VHL,RAF1,PIK3CB, PIK3CA,TFRC,MLH1,BAP1,TET2,FGFR3,PDGFRA, MRPS18C,APC,HMGXB3,CSF1R,PDGFRB,FGFR4, FGF10,ESR1,BYSL,CCND3,SMO,DPP6,EGFR, CDK6,MYC,NRG1,NOTCH1,MLLT3,RP11-145E5.5, JAK2,GNAQ,PTCH1,AR*	13	5 (2.51)	19 (9.55)	175 (87.94)
Leiomyosarcoma	22	105	*FGF8,RET,PTEN,ATM,CADM1,KMT2A,NOTCH2, MCL1,DDR2,CCND1,FGF19,FGF3,MDM4,KRAS, SDCCAG8,CCND2,RP11-611O2.2,MDM2,ARID1A, FGF14,LAMP1,NA,FGF9,FLT1,ALOX5AP,BRCA2,RB1, MYCL,MPL,HPDL,MUTYH,RAD54L,RAD51B,FANCI, TSC2,PALB2,NLRC3,SLX4,CREBBP,CDH1,RP11-525K10.1, RAP1GAP2,RAD51L3-RFFL,ERBB2,BRCA1,TEX14,RPS6KB1, TP53,RBFOX3,BCL2,STK11,NOTCH3,JAK3,TGFBR3,CCNE1, AKT2,ERCC2,PPP1R13L,PIK3CD,GNAS,ERG,ERBB4,BARD1, RNA5SP495,CHEK2,RP1-302D9.3,EP300,RNU6-688P, MSH6,VHL,MKRN2,ATR,MLH1,,TET2,FGF2,FGFR3,PDGFRA, KDR,FGF5,APC,HMGXB3,CSF1R,PDGFRB,FGF10,PIK3R1, DHFR,ROS1,HIVEP1,ESR1,BYSL,MET,SMO,BRAF,DPP6, CARD11,EGFR,CASC11,NRG1,FGFR1,NOTCH1,MLLT3, LINGO2,PTCH1,AR*	13	12 (5.19)	20 (8.66)	199 (86.15)
Inflammatory myofibroblastic tumor	10	8	*FGF19,FGF3,CCNE1,BARD1,FGFR3,FGF5,PDGFRB,SMO*	1	1 (10)		9 (90)

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TABLE S4
Small variants and copy number variants in leiomyoma and leiomyosarcoma samples

Tumor type	Case identification	Small variants				Copy number variants		
		Total small variants, n	Single nucleotide variants, n (%)	Deletions, n (%)	Insertions, n (%)	Total, n	Deletions, n (%)	Duplications, n (%)
Leiomyoma	16LM	20	17 (85)	1 (5)	2 (10)	2	2 (100)	0
	17LM	16	15 (93.75)	0	1 (6.25)	0	0	0
	22LM	12	11 (91.67)	0	1 (8.33)	2	0	2 (100)
	23LM	13	11 (84.62)	0	2 (15.38)	0	0	0
	25LM	37	33 (89.19)	1 (2.7)	3 (8.11)	0	0	0
	28LM	22	21 (95.45)	0	1 (4.55)	1	0	1 (100)
	30LM	17	15 (88.24)	0	2 (11.76)	0	0	0
	32LM	15	13 (86.67)	1 (6.67)	1 (6.67)	8	5 (62.5)	3 (37.5)
	33LM	2	2 (100)	0	0	0	0	0
	34LM	11	9 (81.82)	1 (9.09)	1 (9.09)	2	0	2 (100)
	35LM	18	15 (83.33)	1 (5.56)	2 (11.11)	0	0	0
	36LM	15	12 (80)	0	3 (20)	0	0	0
	41LM	1	1 (100)	0	0	1	0	1 (100)
Leiomyosarcoma	LMS02	41	37 (90.24)	2 (4.88)	2 (4.88)	0	0	0
	LMS03	19	18 (94.74)	0	1 (5.26)	0	0	0
	LMS04	25	21 (84)	2 (8)	2 (8)	0	0	0
	LMS05	15	14 (93.33)	0	1 (6.67)	7	3 (42.8)	4 (57.2)
	LMS06	21	20 (95.24)	0	1 (4.76)	7	6 (85.7)	1 (14.3)
	LMS08	12	9 (75)	1 (8.33)	2 (16.67)	2	0	2 (100)
	LMS09	13	11 (84.62)	2 (15.38)	0	6	0	6 (100)
	LMS10	10	7 (70)	0	3 (30)	1	0	1 (100)
	LMS11	10	10 (100)	0	0	6	6 (100)	0
	LMS12	13	10 (76.92)	1 (7.69)	2 (15.38)	10	6 (60)	4 (40)
	LMS13	17	13 (76.47)	2 (11.76)	2 (11.76)	2	0	2 (100)
	LMS14	27	23 (85.19)	1 (3.7)	3 (11.11)	0	0	0
	LMS15	8	6 (75)	1 (12.5)	1 (12.5)	9	3 (33.3)	6 (66.7)

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(continued)

TABLE S4
Small variants and copy number variants in leiomyoma and leiomyosarcoma samples *(continued)*

Tumor type	Case identification	Small variants				Copy number variants		
		Total small variants, n	Single nucleotide variants, n (%)	Deletions, n (%)	Insertions, n (%)	Total, n	Deletions, n (%)	Duplications, n (%)
Inflammatory myofibroblastic tumor	IMT01[a]	10	9 (90)	1 (10)	0	8	6 (75)	2 (25)

[a] Initially diagnosed as leiomyosarcoma and subsequently confirmed as inflammatory myofibroblastic tumor.

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TABLE S5
Most differentially expressed genes in leiomyosarcoma

Gene	Log fold change	Log counts per million	Likelihood ratio	*P* value	False discovery rate
ALK	2.73	11.87	37.82	7.74E−10	5.42E−09
BRCA2	2.03	11.98	39.86	2.72E−10	2.18E−09
FGFR3	3.41	10.51	35.86	2.12E−09	1.32E−08
FGFR4	3.66	10.33	51.92	5.77E−13	6.46E−12
FLT3	3.92	9.17	26.46	2.68E−07	1.25E−06
NTRK1	3.32	10.05	28.03	1.19E−07	6.08E−07
PAX3	9.67	10.44	63.27	1.8E−15	5.03E−14
PAX7	10.37	10.95	58.60	1.93E−14	2.7E−13
RET	3.07	11.05	42.96	5.56E−11	5.19E−10
ROS1	8.96	10.16	58.99	1.58E−14	2.7E−13
TMPRSS2	8.99	9.56	66.81	2.98E−16	1.67E−14

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