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# Spectrum of mutations in leiomyosarcomas identified by clinical targeted next-generation sequencing☆

Paul J Lee [a,1], Naomi S. Yoo [a,1], Ian S. Hagemann [a], John D Pfeifer [a], Catherine E Cottrell [a,2], Haley J Abel [b], Eric J Duncavage [a,*]

[a] Department of Pathology and Immunology, Washington University, St. Louis, MO, United States
[b] Department of Genetics, Washington University, St. Louis, MO, United States

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ABSTRACT

Recurrent genomic mutations in uterine and non-uterine leiomyosarcomas have not been well established. Using a next generation sequencing (NGS) panel of common cancer-associated genes, 25 leiomyosarcomas arising from multiple sites were examined to explore genetic alterations, including single nucleotide variants (SNV), small insertions/deletions (indels), and copy number alterations (CNA). Sequencing showed 86 non-synonymous, coding region somatic variants within 151 gene targets in 21 cases, with a mean of 4.1 variants per case; 4 cases had no putative mutations in the panel of genes assayed. The most frequently altered genes were *TP53* (36%), *ATM* and *ATRX* (16%), and *EGFR* and *RB1* (12%). CNA were identified in 85% of cases, with the most frequent copy number losses observed in chromosomes 10 and 13 including *PTEN* and *RB1*; the most frequent gains were seen in chromosomes 7 and 17. Our data show that deletions in canonical cancer-related genes are common in leiomyosarcomas. Further, the spectrum of gene mutations observed shows that defects in DNA repair and chromosomal maintenance are central to the biology of leiomyosarcomas, and that activating mutations observed in other common cancer types are rare in leiomyosarcomas.



## 1. Introduction

Sarcomas are mesenchymal malignancies that account for <1% of all adult cancers with fewer than 12,000 cases in the United States diagnosed per year (Siegel et al., 2015). Leiomyosarcomas are malignant smooth muscle tumors that account for <10% of all sarcomas (Goldblum et al., 2014; Gustafson et al., 1992). Despite their relatively low incidence, leiomyosarcomas are associated with poor clinical prognosis due to their characteristic chemoresistance and hematogenous metastatic potential (Weiss, 2002). Although most leiomyosarcomas arise from the uterine myometrium, they can arise in a variety of anatomic sites, including retroperitoneum, extremities, epidermis/dermis, and vasculature (Yang et al., 2009). Leiomyosarcomas are identified histologically by their characteristic morphologic appearance including an elongated, fusiform cytomorphology with abundant pink cytoplasm and blunt-ended nuclei (Goldblum et al., 2014; Yang et al., 2009). Factors such as age (>60 yrs), size (≥5 cm), vascular invasion, location, depth of initial tumor, and stage are independent factors associated with poor prognosis (Weiss, 2002; Miyajima et al., 2002; Wile et al., 1981; Hashimoto et al., 1986). The 5-year overall survival for uterine leiomyosarcoma is 6%–60% depending on AJCC stage at time of diagnosis (Zivanovic et al., 2009).

Despite their aggressive nature, currently little is known about genetic alterations in leiomyosarcomas. Array and cytogenetic based studies of leiomyosarcomas have shown significant molecular heterogeneity (Yang et al., 2009; Guillou and Aurias, 2010; Larramendy et al., 2006; Mertens et al., 1998; Wang et al., 2001) with frequent copy number alterations such as loss of 13q and 10q. These chromosomal areas encompass regions with *RB1* and *PTEN* mutations suggesting their role in tumorigenesis (Yang et al., 2009; Guillou and Aurias, 2010). Other studies have implicated alterations in *TP53*, *MDM2*, *CDKN2A*, *KIT*, *ATRX*, and *MED12* to be associated with leiomyosaroma (Yang et al., 2009; Ravegnini et al., 2013; Grossman et al., 2012; Makinen et al., 2016). Some studies have categorized tumors into subgroups by gene expression profiling or made correlations of specific alterations to prognosis, but currently no molecular biomarkers are used for routine prognostication or treatment determination (Guillou and Aurias, 2010; Beck et al., 2010; Ren et al., 2003). In this study we performed a targeted next generation sequencing (NGS) based panel analysis of common cancer-

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* Corresponding author at: Department of Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8118, St. Louis, MO 63110, United States.
*E-mail address:* EDuncavage@path.wustl.edu (E.J. Duncavage).

[1] These authors contributed equally to this work.

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associated genes to evaluate gene mutations and copy number alterations (CNA) in 25 cases of leiomyosarcomas occurring in a range of anatomic sites.

## 2. Materials and methods

### 2.1. Case selection

Twenty five cases diagnosed as primary or metastatic leiomyosarcoma were selected from samples submitted to Genomics and Pathology Services at Washington University in St. Louis (GPS@WUSTL) between June 2012 and August 2014. The 25 cases (Table 1) were sequenced using the GPS Comprehensive Cancer Panel version 2, comprising 151 total genes (Table S1). Sixteen of the 25 cases (64%) had metastatic disease at the time of study; of these, 12 cases were sequenced from the tumor metastases (48% of total cases). The histologic grading of the leiomyosarcomas was performed using the French Federation of Cancer Centers Sarcoma Group (FNCLCC) system by two board-certified pathologists (Coindre, 2006). Tumor differentiation, mitotic rate and amount of tumor necrosis were used to determine the grade of the leiomyosarcoma. Gynecologic leiomyosarcomas were also graded via this system, although it has not been specifically validated in this context.

The use of human subject material was performed in accordance with guidelines set by the Human Research Protection Office of Washington University.

### 2.2. DNA extraction, capture, and sequencing

Testing was performed on DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue from tumor submitted for clinical NGS. The areas of interest were identified on hematoxylin and eosin-stained slides, selecting for areas of highest tumor cellularity and viability. Tumor cellularity was enriched via macrodissection using sterile 1 mm tissue punches (1–6 cores per tumor). Total genomic DNA was extracted from tissue cores or unstained slides (standard input 750 ng–1 μg; minimum of 100 ng required for testing). DNA was fragmented to a length of 140–230 bp using a Covaris S220 ultrasonicator (Covaris, Woburn, MA), end-repaired, and ligated to universal Illumina sequencing adapters. Target enrichment was performed by custom solution-phase biotinylated cRNA capture baits (SureSelect, Agilent Technologies, Santa Clara, CA) complementary to all coding exons of the targeted genes, up to 50 bp flanking intronic sequence, and selected intronic regions (~800 kb total). Libraries were sequenced to high depth of coverage using an Illumina HiSeq 2500 in rapid run mode (Illumina, Inc., San Diego, CA) with 2 × 101 bp paired-end reads. Sequence reads were aligned to the human reference genome using Novoalign (UCSC build hg19, NCBI build 37.2). Single nucleotide variants (SNV) and indels of sizes >70 bp and between 1 and 20 bp were called using Pindel v0.2.4d and GATKv1.2 unified genotyper respectively. Copy number alterations were called by CopyCat2 on normalized target capture sequences against pooled normal controls. In an effort to remove rare germline polymorphisms, SNV and indel calls were filtered to restrict to non-synonymous variants with a population minor allele frequency of <1% in any population as reported in the NHLBI Exome Variant Server or 1000 Genomes databases (Exome Variant Server, 2014; 1000 Genomes Project Consortium and McVean, 2012).

**Table 1**
Demographics and tumor site distribution.

| | |
|---|---|
| Sex - number (%) | |
| Female | 18 (72) |
| Male | 7 (28) |
| Age at diagnosis - year | |
| Median | 59 |
| Range | 18–79 |
| Age at sequencing - year | |
| Median | 64 |
| Range | 18–79 |
| Tumor location - number (%) | |
| Uterine/pelvic | 9 (36) |
| Retroperitoneal/abdominal | 5 (20) |
| Somatic soft tissue | 7 (28) |
| Vascular | 3 (12) |
| Other | 1 (4) |
| Specimen sequenced - number (%) | |
| Primary | 11 (44) |
| Local recurrence | 2 (8) |
| Distant metastasis | 12 (48) |

## 3. Results

Twenty-five cases of leiomyosarcoma were sequenced including tumors from various anatomic sites with the greatest number of cases arising from uterine/pelvic origin (36%). Patients ranged from 18 to 79 years of age and an approximately 2:1 female predominance (Table 1). Diagnoses were based on standard histopathological criteria in conjunction with immunohistochemical analysis using the WHO classification of soft tissue tumors (Fletcher et al., 2013). The leiomyosarcomas all demonstrated areas with characteristic morphology, including intersecting fascicles of spindled cells with abundant eosinophilic cytoplasmic and elongated nuclei (Fig. 1). 13/25 (52%) cases were classified as high-grade while 8/25 (32%) cases were determined to be intermediate grade using the FNCLCC sarcoma classification (Table S2). There were no examples of low-grade leiomyosarcoma in the cohort.

Following target capture, NGS was performed with a median unique coverage across targeted regions of 793×. Variants identified included SNV, indels, and CNAs. All cases met standard quality assurance metrics for accurate detections of canonical mutations. After filtering and removing duplicate reads, 86 non-synonymous variants within 151 gene targets in 21 cases were identified. The remaining 4 cases had no apparent mutations identified on our somatic gene panel. Seventy-seven (89%) of these variants were interpreted as probable somatic mutations and these variants were not reported in the NHLBI Exome Variant Server, 1000 Genomes, dbSNP vB142, or COSMIC v65: cancer.sanger.ac.uk (Exome Variant Server, 2014; 1000 Genomes Project Consortium and McVean, 2012; Fletcher et al., 2013; Sherry et al., 2001; Forbes et al., 2011). Cases had a mean of 4.1 and median of 3 non-synonymous SNVs and/or small indels per case, excluding polymorphisms (range 0 to 10) (Fig. 2). The most frequent recurrently altered genes included *TP53* (36%), *ATM* and *ATRX* (16%), and *EGFR* and *RB1* (12%) (Table 2). Thirteen of the 86 variants had been previously identified in cancer (COSMIC database) (Sherry et al., 2001), including all *TP53* mutations.

Copy number alterations could be analyzed in only 13 of the 25 leimyosarcomas due to difficulties in normalizing the coverage data in some sequencing runs performed with older versions of sequencing chemistries; 11/13 (85%) cases demonstrated copy number variations (Figs. 3 and 4). Copy number analysis showed that losses were more prevalent than gains with an average of 3.8 losses per case vs 0.5 gains ($p < 0.001$ by unpaired $t$-test) (Table 3). The most frequent copy number losses were observed in chromosomes 13 and 10 (10 and 9 cases, respectively), while the most frequent gains were seen in chromosome 7 and 17 (6 cases) (Fig. 5). The genes with the most frequent deletions were *ABCC2, FGFR2, PTEN, RB1,* and *FLT3*. The most frequently amplified gene was *MAP2K4*.

## 4. Discussion

This study demonstrates the complex genetic aberrations associated with leiomyosarcomas. The most frequently altered gene was *TP53* (36% of cases); all *TP53* variants had been previously documented in the