Research Article

Molecular Analysis of a Recurrent Sarcoma Identifies a Mutation in FAF1

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A patient presented with a recurrent sarcoma (diagnosed as leiomyosarcoma) 12 years after the removal of an initial cancer (diagnosed as extracompartimental osteosarcoma) distally on the same limb. Following surgery, the sarcoma and unaffected muscle and bone were subjected to measurements of DNA exome sequence, RNA and protein expression, and transcription factor binding. The investigation provided corroboration of the diagnosis leiomyosarcoma, as the major upregulations in this tumor comprise muscle-specific gene products and calcium-regulating molecules (calcium is an important second messenger in smooth muscle cells). A likely culprit for the disease is the point mutation S181G in FAF1, which may cause a loss of apoptotic function consecutive to transforming DNA damage. The RNA levels of genes for drug transport and metabolism were extensively skewed in the tumor tissue as compared to muscle and bone. The results suggest that the tumor represents a recurrence of a dormant metastasis from an originally misdiagnosed neoplasm. A loss of FAF1 function could cause constitutive WNT pathway activity (consistent with the downstream inductions of IGF2BP1 and E2F1 in this cancer). While the study has informed on drug transport and drug metabolism pharmacogenetics, it has fallen short of identifying a suitable target for molecular therapy.

1. Introduction

Sarcomas are cancers of mesenchymal origin [1] that comprise about 1% of adult malignancies. Leiomyosarcomas are derived from smooth muscle cells. At most primary sites, other than the uterus or gastrointestinal tract, leiomyosarcomas are likely to originate from the tunica media of blood vessels. However, it has been postulated that primary leiomyosarcoma of the bone might also develop through advanced myogenic metaplasia of a sarcoma originating from fibroblastic tissue [2]. The disease typically occurs in the 5th to 6th decades of life, with women being affected more than men (2:1). This gender distribution may reflect the proliferation of smooth muscle that can occur in response to estrogen [3].

Because sarcomas tend to respond poorly to standard chemotherapy, they have no good treatment options beside total excision with wide margins. However, the gradual replacement of the highly toxic conventional cancer chemotherapy (comprising nonspecific antiproliferative agents) with molecularly targeted drugs, which was initiated with the market entry of neutralizing antibodies and small molecule kinase inhibitors in 1997 (Rituxan) and 2001 (Gleevec), respectively, has opened the possibility to tailor drug treatment to particular tumors. Yet, this transition has also necessitated the molecular characterization of the lesions that are causative for the transformation of healthy cells to cancerous cells because drugs need to be matched with the underlying carcinogenic defect to be effective. Here, we take a sarcoma through a comprehensive molecular analysis that applies multiple screening techniques, with the goal to identify the disease-causing defects as well as potential drug targets.

2. Materials and Methods

2.1. Patient and Tissues. A 52-year-old female patient underwent surgery for a recurrent sarcoma. Samples of skeletal muscle, bone, and tumor were obtained postsurgery.
2.2. DNA Exome Sequencing. 1 μg of dsDNA determined by Invitrogen Qubit high sensitivity spectrofluorometric measurement was sheared by sonication to an average size of 300 bp on a Diagenode Bioruptor. Automated library construction was performed on an IntegenX Apollo324 which size-selects fragments by double-SPIR binding with different concentrations of PEG for a high cut and a low cut. Each library can be fitted with one of 48 adapters, each containing a different 6-base molecular barcode for high level multiplexing. After 12 cycles of PCR amplification, 1 μg of genomic library was recovered for exome enrichment using the NimbleGen EZ Exome V2 kit. Enriched libraries were sequenced on an Illumina HiSeq2000, generating around 32 million high quality paired end reads of 100 base each or 6.4 GB of usable sequence per sample. The analysis methods utilize the Broad Institute’s Genome Analysis Toolkit (GATK) and follow a pipeline previously described [4], along with published modifications (http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit). The analysis comprises aligning the reads that pass Illumina Chastity Filter with the Burrs-Wheeler Aligner (BWA) [5]. For each sample, Picard’s MarkDuplicates are used to flag reads that appear to be artifacts of PCR bias. All reads that overlap known or putative indels are realigned. All base quality scores are recalibrated to the empirical error rate derived from nonpolymorphic sites. The GATK’s Unified Genotyper module is used to call variant sites (both single nucleotide and small indel) in all samples simultaneously. Finally, the SNV calls are filtered using the variant quality score recalibration method [4]. Indel calls were filtered with a set of hard filters, as there are not enough indels in an exome to use the Gaussian method.

2.3. RNAseq. Tissue samples were homogenized in RNazol RT (MRC) with a manual homogenizer and stored on ice until extraction. The RNA isolation was performed according to the manufacturer’s instructions.

The Ovation RNA-Seq FFPE system (NuGen) was used to initiate amplification at both 3’ end as well as randomly throughout the transcriptome in the sample. 100 ng of total RNA with RIN < 5.0 was converted into a library of template molecules suitable for subsequent cluster generation and sequencing by Illumina HiSeq. Total RNA was reverse transcribed and converted to double stranded cDNA with a unique DNA/RNA heteroduplex at one end. NuGen’s Ribo-SPIA technology was used for isothermal amplification resulting in the rapid generation of cDNA with a sequence complementary to the original mRNA. The cDNA was then double stranded and fragmented to 200 bp using Covaris S2, and a sequencing library was generated using Illumina’s TruSeq DNA Sample Prep Kit V2 according to standard protocols. The cDNA library was enriched by a limited number of 10 PCR cycles, validated using an Agilent 2100 Bioanalyzer, and quantitated using the Quant-iT dsDNA HS Kit (Invitrogen). Two individually indexed cDNA libraries were pooled and sequenced on Illumina HiSeq to get a minimum of 90 million reads. Libraries were clustered onto a flow cell using Illumina’s TruSeq SR Cluster Kit v2.5 and sequenced 50 cycles using TruSeq SBS Kit-HS on HiSeq. The obtained sequence reads were aligned to the genome by using the standard Illumina sequence analysis pipeline.

2.4. Protein-DNA Array. Tissues were ground between frosted glass slides and then incubated with Collagenase and Dispase in cell culture medium at 37°C for 45 minutes to release individual cells. These cells were collected after passing the samples through a strainer and centrifugation. Nuclear extracts and cytosol were prepared using a kit from Active Motif. After protein determination, DNA binding of the nuclear extracts was assessed with the Combo protein-DNA array (Panomics). Signal intensity was measured with the software MetaMorph.

2.5. Western Blotting. For the analysis of individual proteins, tissues were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate) using a handheld, battery-operated homogenizer. 10 μg lysates were loaded per lane and electrophoresed on 10% SDS-polyacrylamide minigels with reducing, denaturing sample buffer. The separated proteins were transferred to PVDF membranes and probed with antibody O-17 (IBC) to the C-terminus of osteopontin and anti-H-认知 to the cytoplasmic domain of CD44 (Santa Cruz) and to STAT3 and phospho-STAT3 (Cell Signaling Technology). Antitubulin serves as a loading control.

2.6. 2D Gel Electrophoresis and Mass Spectrometry. The tumor and muscle samples were diluted to 4 and 1 mg/mL in 1:1 diluted SDS Boiling Buffer: Urea Sample Buffer before loading (the bone sample was ethanol precipitated and redissolved to 4 and 1 mg/mL in 1:1 diluted SDS Boiling Buffer: Urea Sample Buffer). Two-dimensional electrophoresis was performed according to the carrier ampholine method of isoelectric focusing [9, 10] by Kendrick Labs, Inc. Isoelectric focusing was carried out in glass tubes of inner diameter 2.3 mm using 2% pH 4–8 Servalytes (Serva, Germany) for 9600 volt-hours. 1 μg (Coomassie stain) or 50 ng (silver stain) of an IEF internal standard, tropomyosin, was added to each sample. This protein migrates as a doublet with lower polypeptide spot of MW 33,000 and pl 5.2; its position is marked by an arrow on the stained gels. The enclosed tube gel pH gradient plot for this set of ampholines was determined with a surface pH electrode.

After equilibration for 10 min in buffer “O” (10% glycerol, 50 mm dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that was on top of a 10% acrylamide slab gels (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 hours at 15 mA/gel. The following proteins (Sigma Chemical Co.) were used as molecular weight standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). These standards appear along the basic edge of the Coomassie blue R-250 stained or silver-stained [11] 10% acrylamide slab gel. The gels were dried between sheets of cellophane paper with the acid edge to the left. Each of the gels was overlaid with a transparent sheet for labeling polypeptide spot differences without marking the original gel (Kendrick Labs).
2.7. Polymorphism Analysis. We obtained 22 formalin-fixed, paraffin-embedded leiomyosarcoma specimens (stroma and paraffin had been removed from unstained slides under microscopic examination) through the Department of Pathology, University of Cincinnati. DNA was extracted with the AllPrep DNA/RNA FFPE kit (Qiagen). We purchased 7 frozen leiomyosarcoma tissues from Creative Bioarray and extracted DNA with the AllPrep DNA/RNA Mini Kit (Qiagen). One blood sample from a leiomyosarcoma patient was received from the University of Cincinnati Tissue Bank. Polymorphisms in FAF1 were analyzed in the DNA using a custom TaqMan assay with the probe ACACCA-GATTTGCCACCACCTCAGCATCT [A/G] GTCAT-GCTGGGTAGTTTTATATTTTCCTG. A TaqMan assay with an existing probe for position −443 in the osteopontin promoter served as a reference assay. 34 breast cancer DNA samples served as nonsarcoma control. The assay was performed by the CCHMC DNA core.

3. Results

3.1. Patient History. In 1998, the patient was diagnosed with high grade, stage IIb osteogenic sarcoma of the right femur, which was extracompartmental. Upon resection the lesion had a size of 9.5 × 3 × 4 cm (Figure 1). The tumor was assessed as stage T2N0Mx, grade III, characterized as hypercellular; it showed marked cytologic atypia and a high mitotic rate. Histologic features included anaplasia, pleomorphism, numerous abnormal mitoses, numerous giant cells, and osteoid production with focal calcifications.

In 2012, a CT scan, done because of hip pain, revealed a 4.3 × 3.4 × 3.1 cm lytic mass in the superior right acetabulum, grossly stable in size and configuration. There was diffuse osteopenia involving the right femoral head and neck with diffuse atrophy of the right pelvic girdle muscle. Periostitis and cortical interruption were associated with this lesion.

The postsurgical pathology report identified the 5.7 × 5.5 × 4.9 cm mass as a high grade leiomyosarcoma, stage pT2bN0X. Whereas a bone scan revealed intense activity in the left seventh rib, a follow-up chest CT provided no indication of pulmonary masses, mediastinal or hilar lymphadenopathy, enlargement of axillary nodes, or pleural effusion, implying stage M0. The mitotic rate was 70%, with 60% necrosis. The tumor caused extensive bone destruction and involvement of adjacent tissue. Histologically, the tumor cells stained positively for smooth muscle actin. They were
also positive for CD68 and displayed diffuse positive staining for vimentin but were negative for CD117, pancreaticin, S-100, and CD34.

Seven months after the surgery, the patient received a PET-MRI scan for pain, which revealed six metastatic lesions, including both lungs and multiple ribs. She was put on three 21-day cycles of Gemzar (days 1 and 8), Taxotere (day 8), and Neulasta (beginning on day 9) but was unable to continue past the first cycle due to hospitalizations for continued and problematic wound infections at the surgical lung biopsy sites.

3.2. DNA Exome Sequence. Exome sequencing of the genomic DNAs for tumor, muscle, and bone identified 65546 potential sequence variants. Filtering yielded 46 likely somatic mutations in the tumor (Table 1), of which 7 (affecting ELF4A1, EPHA3, FAF1, IPO8, KIAA1377, LIMCH1, and NIPBL) were confirmed in the RNASeq results. FAF1 associates with FAS and enhances apoptosis mediated through this receptor [12]. The point mutation S181G (Figure 2) could cause a loss of function in FAF1 and lead to transformation via antiapoptosis.

3.3. RNA Analysis. Expectedly, the gene expression patterns, according to RNASeq, were very different among tumor, muscle, and bone. The cancer contained several gene products that were overexpressed compared to both muscle and bone (Tables 2(a) and 2(b)). Among the top 30 changes in the tumor/muscle and tumor/bone comparisons, 13 were identical (Table 2(c)). It is implied that these gene products are quite unique for the tumor and likely contribute to its pathogenesis. This notion is supported by the upregulation of the smooth muscle gene Ano4, which corroborates the leiomyosarcomatous nature of the cancer. When limiting the analysis to genes expressed at least at the level of 1 unit, the 17 genes overexpressed in the tumor/muscle and tumor/bone comparisons contain several extracellular matrix proteins, implying an active remodeling of the tumor microenvironment (Table 2(d)). By contrast, none of the underexpressed gene products in the tumor/muscle comparison matched the tumor/bone comparison (not shown).

Of note, the RNA level of IGF2BP1 (IMP-1, CRD-BP, and ZBP-1) is highly upregulated in the tumor compared to muscle as well as bone. IGF2BP1 is a RNA-binding factor that affects mRNA nuclear export, localization, stability, and translation. It regulates mRNA stability during the integrated cellular stress response in stress granules. IGF2BP1 is a transcriptional target of the WNT pathway, which is negatively regulated by intact FAP1 and may be unregulated by FAP1 S181G. The IGF system has been linked to sarcoma pathogenesis [13] and may play a role in this specific cancer. Other IGF family members with increased RNA message levels in this tumor (compared to muscle and bone) include IGFBP1 (5-6-log₂-fold), IGFL3 (6-7-log₂-fold), and IGF2BP3 (2-6-log₂-fold).

The identified point mutation in FAF1 may be pathogenic for this cancer. FAF1 is a regulator of NF-κB activation. It directly binds to RelA (P65), retaining it in the cytoplasm. It can also interact with IKKβ, thus allowing for the IκB-mediated degradation of the transcription factors P65 and P50 [6]. Consistently, the expression of regulators of the NF-κB activation pathway is skewed in the tumor compared to muscle or bone (Table 2(e)).

3.4. Transcription Factor Binding. Protein/DNA arrays measure the binding activity of transcription factors. They comprise three basic steps. A set of biotin-labeled DNA binding oligonucleotides are preincubated with a nuclear extract of interest. The protein/DNA complexes are separated from the free probes. The probes in the complexes are then extracted and hybridized to presotted membranes followed by HRP-based chemiluminescence detection. We made nuclear extracts from tumor, bone, and muscle and tested them for DNA binding activity. Binding that was induced in cancer, but not in the normal tissues, was displayed by the transcription factors E2F1, AP3, LIII-BP, PAX6, ADD-1, and CCAC [14–19]. The CCAC binding activity is consistent with a muscle-derived tumor. E2F1 may associate with the WNT pathway-induced transcription factor LEFI, resulting in transcriptional derepression of E2F1 [20]. Likely constitutive transcription factors that are active in all 3 tissues comprise AhR/Amt, GATA1, GATA2, GATA1/2, HIF1, and HOXD8/9/10 (Figure 3).

3.5. Protein Analysis. 2D gel electrophoresis of the RIPA lysates from tumor, muscle, and bone showed very divergent patterns (Figure 4(a)). Two experienced analysts compared the protein pattern from the tumor with the protein pattern from either bone or muscle. Polypeptide spots that were unique to the gels from the tumor were outlined (spots unique to or relatively darker in the bone or muscle were not indicated). The labeled proteins were extracted for identification with mass spectrometry. This yielded several structural proteins, which may reflect modification of the cellular architecture under rapid growth. Transgelin-1 and transgelin-2 were abundant and corroborated the identity of the tumor as a leiomyosarcoma. Four calcium-binding proteins were highly expressed in the cancer. In addition, regulators of protein synthesis (40S ribosomal protein S12, glycine-tRNA ligase), protein modification (N-terminal fragment of heat shock protein HSP 90α, C-terminal fragment of protein disulfide isomerase), and protein degradation (α1-antitrypsin, proteasome activator complex subunit 2) were identified (Figure 4(b)). Of interest may be the C-terminal fragment of protein disulfide isomerase, which not only hydroxylates prolines in preprocollagen but also contributes to microsmal triglyceride transfer. It could be reflective of a skewed tumor metabolism. The protein analysis was corroborated by the mRNA levels (Figure 4(d)).

Cancer markers were tested according to Western blot (Figure 4(c)). The tumor, but not normal muscle, expressed the metastasis protein osteopontin and a single small form (<75 kD) of CD44 that is likely the not alternatively spliced, standard form. Unexpectedly, while both tumor and muscle expressed comparably abundant amounts of STAT3, phosphorylation (reflective of activation) was present in the muscle but not in the tumor. The STAT3 pathway is associated with progression in several human cancers, and this is often
### Table 1: DNA exome SNPs

The DNA exomes for tumor, muscle, and bone were sequenced. The results were filtered in the following order: (1) different genotype in tumor from muscle and bone, with muscle and bone being identical to each other, (2) deletion mutations with low confidence (value of 20 or lower) in all 3 tissues, (3) delete unidentified genes, (4) delete mutations that are homozygous reference in the tumor, (5) delete mutations that have a MAF in dbSNP >10%, (6) delete low impact and modifier mutations. The gene names are part of the key in the left column. In this column, results on bold font represent SNPs that were confirmed on the RNA level by RNASeq. The data files have been submitted to the NCBI short read archive (SRA) under the accession number SRP052797 (biosamples SAMN03316820, SAMN03316821, SAMN03316822).

| Key | Chromosome | Position | Reference | Alternate | dbSNP ID | dbSNP MAF | ESP MAF (AA) | ESP MAF (All) | Consensus allele | Bone genotype | Bone overall depth | Bone allele depths | Muscle genotype | Muscle overall depth | Muscle allele depths | Tumor genotype | Tumor overall depth | Tumor allele depths | Tumor quality |
|-----|-------------|----------|-----------|-----------|-----------|-----------|-------------|--------------|----------------|---------------|----------------|----------------|----------------|----------------|----------------|-------------|----------------|----------------|----------|
| 117 | 7479998     | C        | T         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 3   | 89350000    | C        | T         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 1    | 5204545     | C        | T         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 12   | 10834425    | G        | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 11   | 4168282     | G        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 1    | 3698532     | A        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 12    | 77623789    | A        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 2    | 2D300095    | A        | S_MARCALI |         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 2    | 71236045    | T         | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 5    | 52030669    | T        | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 9    | 12130731    | C        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 6    | 1203054     | A         | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 5    | 1208759     | G        | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 7    | 1234860     | G        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 4    | 4929212     | T         | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 11    | 5448032     | A        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 10   | 1689325     | G        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 7    | 4148985     | 1G        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 12    | 5456894     | G        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 20   | 6762155     | A        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 16    | 12703526    | G        | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 7    | 1598808D    | A        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 15    | 4564426     | A        | G         | .         | 0.0009    | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 14    | 8637324     | G        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 7    | 4203393     | G        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 17    | 4807170     | C        | T         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 14    | 2403801     | T        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 15    | 4213306     | G        | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 20    | 4203309     | G        | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 17    | 7427167     | A        | K_AT2A   |         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 8    | 7384894     | L_KCN2    | A         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 19    | 5087053     | C        | T         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 2    | 2109069     | G        | A         | .         | 0.0265958 | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 5    | 1001257     | A        | C         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 10    | 9005840     | C        | P_LCE1    |         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
| 16    | 18816805    | C        | G         | .         | .         | .         | .            | .             | .               | .             | .             | .              | .               | .              | .             | .           | .              | .             | .         |
Table 1: Continued.

| Key | Chromosome | Position | Reference | Alternate | dbsNP ID | dbsNP MAF (All) | ESP MAF (EA) | ESP MAF (AA) | Consensus impact | Bone/ Muscle genotypes | Bone overall depths | Bone allele depths | Bone quality | Muscle overall depths | Muscle allele depths | Muscle quality | Tumor overall depths | Tumor allele depths | Tumor quality |
|-----|------------|----------|-----------|-----------|----------|----------------|--------------|--------------|------------------|----------------------|-------------------|-----------------|-------------|-------------------|-----------------|---------------|-------------------|----------------|-------------|
| 12, ID020739, T_PPTC7 | 12 | 18020739 | TCGC | T | rs7083012 | — | — | — | — | Moderate | 0/0 | 18 | NA | 30 | 14 | NA | 18 | 0/1 | 19 | NA | 10 |
| 19, 804293, A_PBP3 | 19 | 804293 | C | A | . | — | — | — | — | Moderate | 0/0 | 120 | 126 | 99 | 71 | 74 | 99 | 0/1 | 75 | 53 | 99 |
| 7, 15645175, C_RNF63 | 7 | 15645175 | G | C | . | — | — | — | — | Moderate | 0/0 | 55 | 57 | 99 | 67 | 70 | 99 | 0/1 | 59 | 42 | 99 |
| 3, 52020669, T_RH1-E1D18.11 | 3 | 52020669 | G | T | . | — | — | — | — | Moderate | 0/0 | 110 | 116 | 99 | 86 | 90 | 99 | 0/1 | 80 | 45 | 99 |
| 21, 43638624, ZEB1-ISHA | 21 | 43638624 | G | T | . | — | — | — | — | Moderate | 0/0 | 70 | 73 | 99 | 45 | 47 | 99 | 0/1 | 89 | 76 | 99 |
| 19, 5677387, ZNF444 | 19 | 5677387 | G | A | . | — | — | — | — | Moderate | 0/0 | 46 | 48 | 99 | 43 | 45 | 99 | 0/1 | 71 | 51 | 99 |
| 5, 1646572, ZFHB | 5 | 1646572 | T | C | . | — | — | — | — | Moderate | 0/0 | 87 | 170 | 39 | 14 | 14 | 33 | 0/1 | 27 | 14 | 99 |
Table 2: RNA induced in the tumor. (a) Transcripts overexpressed in the tumor compared to muscle but not bone. (b) Transcripts overexpressed in the tumor compared to bone but not muscle. (c) Transcripts most abundantly overexpressed in the tumor compared to muscle and bone. (d) as (a) but limited to genes expressed at least at the level of 1 unit in the reference tissue (muscle or bone). (e) Altered expression of genes associated with NF-κB activity. Analysis of RNASeq for the transcription factor NF-κB and gene products that regulate its activity. RNA messages that are selectively associated with the TNF pathway to NF-κB activation are marked with bold font. log₂-fold change indicates the alteration in the tumor compared to muscle or bone (the respective columns indicate the expression level for each organ).

| Gene Symbol | Gene Name | log₂-fold change |
|-------------|-----------|------------------|
| NRG1        | Neuregulin 1 | 9.909            |
| KSR2        | Kinase suppressor of ras 2 | 9.675            |
| MMP13       | Matrix metalloproteinase 13 (collagenase 3) | 9.528            |
| SPP1        | Secreted phosphoprotein 1 | 9.098            |
| INHBA       | Inhibin, beta A | 8.570            |
| COL11A1     | Collagen, type XI, alpha 1 | 8.564            |
| MMP9        | Matrix metalloproteinase 9 (gelatinase B, 92 kda gelatinase, 92 kda type IV collagenase) | 8.552            |
| FBN2        | Fibrillin 2 | 8.495            |
| LRRCD5      | Leucine rich repeat containing 15 | 8.429            |
| MMP11       | Matrix metalloproteinase 11 (stromelysin 3) | 8.336            |
| E2F7        | E2F transcription factor 7 | 8.314            |
| PRAME       | Preferentially expressed antigen in melanoma | 8.179            |
| PTK7        | PTK7 protein tyrosine kinase 7 | 7.996            |
| DSCAM       | Down syndrome cell adhesion molecule | 7.761            |
| RGS4        | Regulator of G-protein signaling 4 | 7.633            |
| CNH3        | Cornichon homolog 3 (Drosophila) | 7.632            |
| OR10V1      | Olfactory receptor, family 10, subfamily V, member 1 | 7.610            |
| CEP55       | Centrosomal protein 55 kda | 7.583            |
| WNT5B       | Wingless-type MMTV integration site family, member 5B | 7.569            |
| CA12        | Carbonic anhydrase XII | 7.520            |
| GALNT5      | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5) | 7.517            |

| Gene Symbol | Gene Name | log₂-fold change |
|-------------|-----------|------------------|
| ROS1        | c-ros oncogene 1, receptor tyrosine kinase | 9.987            |
| GREM1       | Gremlin 1 | 10.604           |
| NPTX1       | Neuronal pentraxin 1 | 8.813            |
| GJB2        | Gap junction protein, beta 2, 26 kDa | 8.597            |
| CREB3L1     | cAMP responsive element binding protein 3-like 1 | 8.506            |
| KRT14       | Keratin 14 | 8.351            |
| SERPINE1    | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 8.288            |
| HOXD10      | Homeobox D10 | 8.105            |
| ALPK2       | Alpha-Kinase 2 | 7.783            |
| POU3F2      | POU class 3 homeobox 2 | 7.530            |
| POSTN       | Periostin, osteoblast specific factor | 7.497            |
| NAA11       | N(alpha)-acetyltransferase 11, NatA catalytic subunit | 7.439            |
| STC2        | Stanniocalcin 2 | 7.434            |
| WNT5A       | Wingless-type MMTV integration site family, member 5A | 7.412            |
| IGFN1       | Immunoglobulin-like and fibronectin type III domain containing 1 | 7.387            |
### (b) Continued.

| Symbol | Name                                                                 | log$_2$-fold change |
|--------|----------------------------------------------------------------------|---------------------|
| STC1   | Stanniocalcin 1                                                   | 7.385               |
| FAM180A| Family with sequence similarity 180, member A                     | 7.315               |
| KRT17  | Keratin 17                                                        | 7.305               |
| BBOX1  | Butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1 | 7.277               |
| MAGEA1 | Melanoma antigen family A, 1 (directs expression of antigen MZ2-E) | 7.267               |

### (c)

| Symbol | Name                                                                                                                               | log$_2$-fold change |
|--------|-------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| ANO4   | Anoctamin 4 (TMEM16D), transmembrane calcium-activated chloride channel, facilitates smooth muscle contraction                     | 9.937 8.542         |
| SLCO1B3| (OATP1B3) Solute carrier organic anion transporter family, member IB3                                                             | 9.569 9.760         |
| MARCH4 | Membrane-associated ring finger (C3HC4) 4, E3 ubiquitin ligase, located predominantly to the endoplasmic reticulum              | 9.538 7.406         |
| IGF2BP1| Insulin-like growth factor 2 mRNA binding protein 1 binds to and stabilizes mRNA                                                | 9.440 9.631         |
| ADAMTS16| ADAM metallopeptidase with thrombospondin type 1 motif, 16; zinc-dependent protease                                             | 9.260 8.450         |
| SOX11  | SRY (sex determining region Y)-box 11, important in brain development                                                            | 9.178 9.954         |
| HAPLN1 | Hyaluronan and proteoglycan link protein 1 stabilizes aggregates of aggrecan and hyaluronan, giving cartilage its tensile strength and elasticity | 8.951 8.142         |
| MUC15  | Mucin 15, cell surface associated                                                                                                 | 8.801 7.992         |
| HOXB9  | Homeobox B9                                                                                                                      | 8.773 7.378         |
| MAGEC2 | Melanoma antigen family C, 2                                                                                                     | 8.693 8.884         |
| ST6GALNAC5| ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5                  | 8.748 8.038         |
| C11orf41| Chromosome 11 open reading frame 41                                                                                                | 7.781 8.141         |
| MMP1   | Matrix metallopeptidase 1 (interstitial collagenase)                                                                               | 7.455 7.646         |

### (d)

| Symbol | Name                                                                 | log$_2$-fold change |
|--------|----------------------------------------------------------------------|---------------------|
| FN1    | Fibronectin 1                                                      | 7.328 6.293 6.457 19.860 |
| COL1A1 | Collagen, type I, alpha 1                                           | 6.768 3.706 5.868 11.934 |
| CCND1  | Cyclin D1                                                          | 5.595 3.958 5.098 9.637 |
| RGS1   | Regulator of G-protein signaling 1                                  | 5.118 1.056 4.653 2.533 |
| ITGBL1 | Integrin, beta-like 1 (with EGF-like repeat domains)                | 5.071 3.177 3.895 12.415 |
| COL1A2 | Collagen, type I, alpha 2                                           | 4.852 8.756 4.910 14.503 |
| MXRA5  | Matrix-remodelling associated 5                                     | 4.834 1.352 4.631 2.685 |
| POSTN  | Periostin, osteoblast specific factor                                | 4.513 5.870 7.497 1.267 |
| PLOD2  | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2                  | 4.285 1.801 4.023 3.730 |
| COL5A2 | Collagen, type V, alpha 2                                           | 4.275 1.297 4.833 1.517 |
| COL3A1 | Collagen, type III, alpha 1                                         | 4.003 13.118 5.801 6.500 |
| SEMA3C | Sema domain, immunoglobulin domain (Ig), short basic domain, secreted | 3.954 1.164 4.215 1.675 |
| FBN1   | Fibrillin 1                                                         | 3.912 6.957 4.018 11.148 |
| AEBP1  | AE binding protein 1                                                | 3.808 3.212 4.002 4.843 |
| SIK1   | Salt-inducible kinase 1                                             | 3.805 1.219 3.566 2.484 |
## (d) Continued.

| Symbol  | Name                                                                 | log$_2$-fold change Muscle | log$_2$-fold change Bone |
|---------|----------------------------------------------------------------------|---------------------------|-------------------------|
| SERPINH1 | Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 | 3.706                     | 4.145                   |
| ANTXR1  | Anthrax toxin receptor 1                                             | 3.670                     | 3.690                   |

## (e)

| Symbol  | Name                                                                 | log$_2$-fold change Muscle | log$_2$-fold change Bone |
|---------|----------------------------------------------------------------------|---------------------------|-------------------------|
| HELLs   | Helicase, lymphoid-specific                                          | 5.315602                  | 0.155898                |
| TNFRSF1A| Tumor necrosis factor receptor superfamily, member 1a, and NFKB activator | 3.017922                  | 0.03091                |
| NFKBID  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta | 2.65352                  | 0                      |
| TNFRSF25| Tumor necrosis factor receptor superfamily, member 25                | 2.432959                  | 0.046388            |
| NFKBIE  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon | 2.121015                 | 0.062395            |
| TNF     | Tumor necrosis factor                                               | 1.847997                  | 0                      |
| TNFRSF10D| Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain | 1.754888                  | 0.172968            |
| TNFRSF1B| Tumor necrosis factor receptor superfamily, member 1B               | 1.473438                  | 0.709902            |
| TNFRSF10A| Tumor necrosis factor receptor superfamily, member 10a             | 1.411898                  | 0.828219            |
| NFKBIB  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta | 1.193993                  | 1.209816            |
| TNFRSF10B| Tumor necrosis factor receptor superfamily, member 10b             | 1.094157                  | 1.908597            |
| TNFRSF21| Tumor necrosis factor receptor superfamily, member 21              | 1.055762                  | 3.882038            |
| TNFRSF1A| Tumor necrosis factor receptor superfamily, member 1A              | 0.875167                  | 3.799093            |
| NFKBIZ  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta | 0.575974                  | 3.698951            |
| RIPK1   | Receptor (TNFRSF)-interacting serine-threonine kinase 1            | 0.494467                  | 3.11749              |
| NIKRF   | NFKB repressing factor                                              | 0.475722                  | 2.156087            |
| NFKB1   | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | 0.432959                  | 2.054332            |
| CHUK    | Conserved helix-loop-helix ubiquitous kinase                       | 0.176126                  | 2.961281            |
| RELA    | V-rel reticuloendotheliosis viral oncogene homolog A (avian)         | 0.013848                  | 1.263837            |
| NFKB2   | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) | -0.08641                  | 0.312993            |
| NFKAPPI | NFKB activating protein pseudogene 1                                | -0.10692                  | 0.825177            |
| TNFRSF10C| Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain | -0.152                   | 0.064676            |
| NKIRAS2 | NFKB inhibitor interacting Ras-like 2                               | -0.60768                  | 1.095135            |
| NFKBIL1 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 | -0.8719                   | 0.141933            |
| NKIRAS1 | NFKB inhibitor interacting Ras-like 1                               | -0.87428                  | 6.296399            |
| TANK    | TRAF family member-associated NFKB activator                        | -0.983                    | 6.777124            |
| NFKAP   | NFKB activating protein                                             | -1.35735                  | 14.22458            |
| NFKBIA  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | -1.85483                  | 40.32743            |
| NFKAPL  | NFKB activating protein-like                                        | -5.57347                  | 5.875743            |
Figure 2: FAF1 structure. (a) A schematic of functional domains in FAF1 with annotations (adapted from [6–8]). There are two ubiquitin-like domains flanking the S181 site. The PDB structure 2DZM identifies the N-terminal one, while the C-terminal prediction is by sequence similarity. Whereas the mutation S181G is not expected to disrupt the protein structure per se, this amino acid has a high score as a possible phosphorylation site for a number of kinases involved in DNA damage repair. (b) Secondary structure prediction of FAF1. The residues around S181 appear unstructured.

reflected in STAT3 constitutive phosphorylation. The lack of phosphorylation in this case suggests that the leiomyosarcoma may not depend on the STAT3 pathway.

3.6. Pharmacogenetic Evaluation. Predicting the sensitivity to anticancer drugs is a main goal of molecular analysis. For this, the over- or underexpression of genes for drug transport and metabolism is of key importance. Analysis of the RNASeq data for these groups of gene products identified a surprisingly large number of deregulations compared to muscle or bone (Tables 3(a) and 3(b)). Those alterations may affect choices for drug treatment. For example, the high levels of glutathione S-transferase may render carmustine, thioTEPA, cisplatin, chlorambucil, melphalan, nitrogen mustard, phosphoramide mustard, acrolein, or steroids ineffective. The overexpression of N-acetyltransferase may compromise 5-fluorouracil or taxol. The modest upregulation of only two export transporters (ABC-transporters), and specifically
3.7 Population Analysis. The above-described results indicated that a FAF1 mutation, which replaces serine in position 181, thus preventing FAF1 phosphorylation and activation, may be a driver for leiomyosarcomagenesis. A custom TaqMan assay confirmed the presence of the somatic mutation in the patient. To assess whether this single nucleotide replacement is common in this type of cancer, we analyzed DNA from 29 leiomyosarcomas and 1 blood sample from a leiomyosarcoma patient. For comparison to nonsarcomatous tumors, 34 breast cancers served as a reference. None of them displayed a mutation in the same locus. By contrast, there was a distribution across all leiomyosarcomas in the osteopontin promoter position – 443 (used as a reference), with 12 CC, 10 TC, and 9 TT.

4. Discussion

The FAF1 mutation identified as the likely cause for the cancer under study gives room for an explanation of the sarcomatous transformation (Figure 5). DNA damage to mesenchymal cells occurs persistently in an oxidizing environment at 37°C. These insults are rarely transforming, and such an occurrence would trigger the initiation of programmed cell death in apoptosis-competent cells. Intact FAF1 associates with FAS and enhances apoptosis mediated through this receptor [12]. A loss of function in FAF1 could lead to transformation via antiapoptosis. Whereas the mutation S181G is not expected
| Spot number | Protein identified                          | SwissProt or NCBI accession | Description                                                                 |
|-------------|--------------------------------------------|-----------------------------|-----------------------------------------------------------------------------|
| 6           | Transgelin                                 | Q01995                      | Smooth muscle-specific, cross-links actin                                  |
| 24          | Transgelin-2                               | P37802                      | Smooth muscle-specific, cross-links actin                                  |
| 11, 15–17   | Vimentin                                   | P08670                      | Intermediate filament in mesenchymal tissue                                |
| 1           | Filamin-A                                  | P21333                      | Actin-binding protein, regulates the cytoskeleton                           |
| 21          | β-actin (C-terminal fragment)              | P06709                      | Cytoskeleton                                                                |
| 8, 9        | Calumenin                                  | O43852                      | Calcium-binding protein in the ER and golgi apparatus                       |
| 26          | Protein S100-A11                           | P31949                      | Calcium-binding EF hand protein                                            |
| 10          | Reticulocalbin-3                           | Q96D15                      | Calcium-binding protein located in the ER                                    |
| 12          | 78 kD glucose-regulated protein            | P11021                      | Heat shock 70-kD protein 5, ER luminal, calcium-binding                     |
| 25          | 40S ribosomal protein S12                  | P25398                      | Core component of the ribosomal accuracy center                            |
| 7           | Glycine-tRNA ligase                        | P41250                      | Catalyzes the attachment of glycine to tRNA                                |
| 19          | α-1-antitrypsin                            | P01009                      | Protease inhibitor                                                          |
| 23          | α-1-antitrypsin (C-terminal fragment)      | P01009                      | Protease inhibitor                                                          |
| 21          | Proteasome activator complex subunit 2     | Q9UL46                      | Proteasome activation                                                       |
| 10          | Heat shock protein HSP 90α (N-terminal fragment) | P07900                | Chaperone                                                                  |
| 7           | Serum albumin                              | P02768                      | Carrier protein in blood                                                    |
| 22          | Protein disulfide isomerase (C-terminal fragment) | P07237                | Prolyl hydroxylation in collagen, microsomal triglyceride transfer          |

**Figure 4:** Continued.
Figure 4: Protein overexpression. (a) 2D protein gel electrophoresis of lysates from muscle (upper left), tumor (upper right), and bone (bottom) in RIPA buffer. Red circles indicate the spots that were identified as overexpressed and were further analyzed by mass spectrometry. (b) Proteins identified in 2D gel electrophoresis as overexpressed in the tumor in comparison to muscle and bone were analyzed for their identity by mass spectrometry. The left column indicates the spot number corresponding to the 2D gel. The next column contains the protein name, followed by the accession number and a description of the protein function. The protein functions are grouped into structural, calcium homeostasis, and various others. (c) Western blot. 10 µg lysates of tumor, muscle, and bone in RIPA buffer were loaded per lane and electrophoresed on 10% SDS-polyacrylamide minigels with reducing, denaturing sample buffer. After transfer to PVDF membranes, they were probed for markers of cancer progression, including osteopontin, CD44, STAT3, and phospho-STAT3. Antitubulin served as a loading control. (d) RNA levels corresponding to the proteins found to be affected in the sarcoma. With four exceptions in the tumor-bone comparison (gray font), upregulated proteins are associated with increased RNA levels. STAT3 is not increased on the protein or RNA level. The reduced level of RB1 expression is consistent with the elevated DNA binding activity of E2F1.

to disrupt the structure of the protein, this site does score high as a possible phosphorylation site for a number of kinases involved in DNA damage repair, supporting the hypothesis that the cancer cells containing this mutation have lost their ability to respond to transforming DNA damage with programmed cell death. FAF1 antagonizes WNT signaling by promoting β-catenin degradation in the proteasome [21], a function that may be lost after the point mutation. The elevated DNA binding activity of the protooncogenic transcription factor E2F1 (see Figure 3) could be caused by its interaction with LEF-1 [20], consecutive to persistent WNT signaling. The RNA level of IGF2BP1 (IMP-1), a stress-responsive regulator of mRNA stability, is highly upregulated in the tumor compared to muscle as well as bone (see Table 2(c)). IGF2BP1 is a transcriptional target of the WNT pathway that regulates NF-κB activity (see Table 2(e)) and is antiapoptotic [22, 23]. IGF2BP1 may be upregulated as a consequence of mutated FAF1 not being able to suppress WNT signaling in this specific cancer. Of note, WNT pathway overactivity may not be required for transformation; rather the persistence of a WNT pathway signal due to the lack of a FAF1-mediated termination signal may suffice.
The role of WNT signaling in sarcoma has been subject to debate (e.g., [24]). In metastatic leiomyosarcoma, β-Catenin may accumulate in the nucleus despite a relatively weak expression of WNT [25]. This may be due to WNT signal activation via noncanonical ligands [26] or to β-Catenin binding the nuclear receptor NR4A2 and releasing it from the corepressor protein LEF-1 [27]. Of note, in the cancer under study here, the mRNA level of NR4A2 was overexpressed 10-fold compared to bone and 3-fold compared to muscle. The results from this study are consistent with the possibility that a lack of termination in the WNT signal, rather than its overactivation, could contribute to sarcomatous transformation. Such a mechanism may be reflected in an upregulation of downstream targets, even though overexpression of WNT pathway components is not detectable.

Other mutations, beside FAF1, are less likely to be causative for the cancer. EPHA3 was revealed as mutated in this cancer by DNA exome sequencing and RNASeq. EPHA3 is a receptor tyrosine kinase that is frequently mutated in lung cancer. Tumor-suppressive effects of wild-type EPHA3 can be overridden by dominant negative EPHA3 somatic mutations [28]. This mechanism is unlikely to play a role in this sarcoma as the detected mutation is located far N-terminally on the extracellular Ephrin binding domain not on the intracellular kinase domain.

FAFI has been described to act as a tumor suppressor gene [29]. Its depletion due to chromosome breakage can affect prognosis in glioblastoma patients [30, 31]. Single nucleotide polymorphisms in FAF1 are associated with a risk for gastric cancer [32]. While numerous FAF1 mutations are associated with various cancers, none of these genetic changes in the TCGA database affects the amino acid position 181 (Table 4).

The major upregulations identified in this tumor comprise muscle-specific gene products (transcription factors: CAAC binding; proteomics: transgelin, transgelin-2; RNA: anoctamin-4; and immunohistochemistry: smooth muscle actin) and calcium-regulating molecules (proteomics: calumenin, SI00-A11, reticulocalbin-3, and 78 kD glucose-regulated protein). The muscle-specific gene products confirm this recurrent sarcoma as a leiomyosarcoma (the first tumor, distal to the site of the recurring one, had been diagnosed as an osteosarcoma). Calcium is one of the major second messengers in smooth muscle cells. Its uptake is regulated by potential-sensitive ion channels in the cell membrane and by the activities of various receptors. Calcium is stored in the sarcoplasmic reticulum, from where it can be released to facilitate actin-myosin interaction and tension generation. Phosphorylation of the myosin light chain by a calmodulin-regulated enzyme is important for contraction. The upregulation of gene products associated with migration and invasion (osteopontin, MMP1, vimentin, filamin-A, and β-actin) and gene products for extracellular matrix molecules and their modulators (fibronectin, collagen, ITGBL1, and MXRA5) reflects the invasive nature of this cancer.

The recurrence of a sarcoma after 14 years has two probable explanations, either it is due to a cancer predisposition syndrome based on a germ-line mutation (the age of the patient weakens this hypothesis) or the second tumor is a metastatic colony of the first that was reactivated after dormancy. The location of the sarcoma in the same extremity and proximal to a preceding mesenchymal cancer (and therefore in its natural path of dissemination) implied the probability that this was a relapse in a metastatic site. The different histologic assessment as osteosarcoma in the first occurrence and leiomyosarcoma as the second cancer does not necessarily negate that. Mixed histology [33, 34] and transdifferentiation [35–37] have been described for sarcomatous tumors. Of note, however, in this scenario osteosarcoma seems to more commonly follow leiomyosarcoma than precede it. Material from the first cancer of this patient was not accessible to us. It is very plausible that this could have been a mineralized leiomyosarcoma. In those tumors, the differential diagnosis from osteosarcoma can be difficult [38]. The extracompartmental location of the first tumor supports this interpretation.

On the molecular pathology level, sarcomas fall into two groups, comprising tumors with simple karyotypes (with pathogenetic translocations or specific genetic mutations) and tumors with very complex karyotypes (overt chromosome and genomic instability with numerous gains and losses) [39]. Some molecular alterations that lead to carcinogenesis can be defined in absolute terms. They include gain-of-function mutations or chromosome translocations that transform protooncogenes to oncogenes. However, other changes are relative to the normal tissue of origin, such as pathway overactivity or overexpression on the protein or RNA levels. We have combined the analysis of absolute changes (DNA exome sequence, RNA sequence) with the analysis of relative changes using skeletal muscle and bone as reference organs (protein-DNA array, 2D gel electrophoresis, and RNA expression levels). This choice was determined in part by tissue availability after surgery and was intended to aid in the distinction of osteosarcoma from myosarcoma. While
Table 3: Deregulation of genes for drug disposition. Analysis of RNASeq for at least twofold overexpression (italic font) or underexpression (bold font) of genes for (a) transport and (b) metabolism in tumor compared to muscle and bone. For the export transporters (ABC transporters), only overexpression is considered relevant for drug resistance. Not shown in the table are the underexpressed genes (ABCA7, ABCA8, ABCA13, ABCB6, ABCB10, ABCC6, ABCC6P1, ABCC6P2, ABCC8, ABCC11, ABCD2, ABCG2, and ABCG5).

(a) Transport

| Gene ID | Symbol   | Name                                                                 | Tumor bone log₂-fold change | Tumor muscle log₂-fold change |
|---------|----------|----------------------------------------------------------------------|-----------------------------|-------------------------------|
| 650655  | ABCA17P  | ATP-binding cassette, subfamily A (ABC1), member 17, pseudogene      | 1.360                       | 2.116                         |
| 24      | ABCA4    | ATP-binding cassette, subfamily A (ABC1), member 4                   | 2.038                       | 2.802                         |
| 6555    | SLC10A2  | Solute carrier family 10 (sodium/bile acid cotransporter family), member 2 | −1.962                     | −2.112                        |
| 345274  | SLC10A6  | Solute carrier family 10 (sodium/bile acid cotransporter family), member 6 | 2.623                       | 3.240                         |
| 6563    | SLC14A1  | Solute carrier family 14 (urea transporter), member 1 (Kidd blood group) | −3.074                     | −3.152                        |
| 6565    | SLC15A2  | Solute carrier family 15 (H⁺/peptide transporter), member 2           | −2.027                      | −2.152                        |
| 6566    | SLC16A1  | Solute carrier family 16, member 1 (monocarboxylic acid transporter 1) | 1.401                       | 2.170                         |
| 117247  | SLC16A10 | Solute carrier family 16, member 10 (aromatic amino acid transporter) | 2.113                       | 2.848                         |
| 6567    | SLC16A2  | Solute carrier family 16, member 2 (monocarboxylic acid transporter 8) | 3.242                       | 3.848                         |
| 10786   | SLC17A3  | Solute carrier family 17 (sodium phosphate), member 3                | −2.868                      | −2.959                        |
| 6571    | SLC18A2  | Solute carrier family 18 (vesicular monoamine), member 2             | −2.087                      | −2.194                        |
| 6573    | SLC19A1  | Solute carrier family 19 (folate transporter), member 1              | −2.099                      | −2.203                        |
| 10560   | SLC19A2  | Solute carrier family 19 (thiamine transporter), member 2             | 1.820                       | 2.548                         |
| 80704   | SLC19A3  | Solute carrier family 19, member 3                                   | −3.331                      | −3.478                        |
| 387775  | SLC22A10 | Solute carrier family 22, member 10                                   | 5.711                       | 6.085                         |
| 9390    | SLC22A13 | Solute carrier family 22 (organic anion transporter), member 13       | 2.623                       | 3.217                         |
| 85413   | SLC22A16 | Solute carrier family 22 (organic cation/carnitine transporter), member 16 | −8.101                     | −7.299                        |
| 51310   | SLC22A17 | Solute carrier family 22, member 17                                   | 1.475                       | 2.175                         |
| 5002    | SLC22A18 | Solute carrier family 22, member 18                                   | 2.038                       | 2.722                         |
| 6582    | SLC22A2  | Solute carrier family 22 (organic cation transporter), member 2       | 4.793                       | 5.216                         |
| 6581    | SLC22A3  | Solute carrier family 22 (extraneuronal monoamine transporter), member 3 | 2.554                       | 3.182                         |
| 6583    | SLC22A4  | Solute carrier family 22 (organic cation/ergothioneine transporter), member 4 | −3.603                     | −3.776                        |
| 151295  | SLC23A3  | Solute carrier family 23 (nucleobase transporters), member 3          | 2.109                       | 2.848                         |
| 10478   | SLC25A7  | Solute carrier family 25 (mitochondrial carrier), member 17           | 1.311                       | 2.077                         |
| 83733   | SLC25A18 | Solute carrier family 25 (mitochondrial carrier), member 18          | 1.846                       | 2.570                         |
| 788     | SLC25A20 | Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20 | −2.105                     | −2.207                        |
| 89874   | SLC25A21 | Solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21 | −2.962                     | −3.105                        |
| 51312   | SLC25A37 | Solute carrier family 25, member 37                                  | −4.633                      | −4.866                        |
| 51629   | SLC25A39 | Solute carrier family 25, member 39                                  | −2.585                      | −2.737                        |
| 203427  | SLC25A43 | Solute carrier family 25, member 43                                  | 1.325                       | 2.088                         |
| 65012   | SLC26A10 | Solute carrier family 26, member 10                                  | 3.896                       | 4.472                         |
| 11511   | SLC26A7  | Solute carrier family 26, member 7                                   | 3.431                       | 4.018                         |
| 116369  | SLC26A8  | Solute carrier family 26, member 8                                   | −5.354                      | −5.536                        |
| 115019  | SLC26A9  | Solute carrier family 26, member 9                                   | 1.623                       | 2.419                         |
| 11001   | SLC27A2  | Solute carrier family 27 (fatty acid transporter), member 2          | −4.278                      | −4.487                        |
| Gene ID | Symbol | Name                                                                 | Tumor bone log₂-fold change | Tumor muscle log₂-fold change |
|---------|--------|----------------------------------------------------------------------|-----------------------------|-----------------------------|
| 64078   | SLC28A3| Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3 | -4.543                      | -4.812                      |
| 222962  | SLC29A4| Solute carrier family 29 (nucleoside transporters), member 4           | -1.962                      | -2.045                      |
| 81031   | SLC2A10| Solute carrier family 2 (facilitated glucose transporter), member 10  | 2.532                       | 3.170                       |
| 6518    | SLC2A5 | Solute carrier family 2 (facilitated glucose/fructose transporter), member 5 | -3.647                      | -3.821                      |
| 55532   | SLC30A10| Solute carrier family 30, member 10                                   | -3.547                      | -3.725                      |
| 7782    | SLC30A4| Solute carrier family 30 (zinc transporter), member 4                 | 2.832                       | 3.372                       |
| 6569    | SLC34A1| Solute carrier family 34 (sodium phosphate), member 1                 | -4.716                      | -4.941                      |
| 340146  | SLC35D3| Solute carrier family 35, member D3                                   | -5.662                      | -5.906                      |
| 54733   | SLC35F2| Solute carrier family 35, member F2                                   | 1.433                       | 2.170                       |
| 206358  | SLC36A1| Solute carrier family 36 (proton/amino acid symporter), member 1       | -2.265                      | -2.345                      |
| 285641  | SLC36A3| Solute carrier family 36 (proton/amino acid symporter), member 3       | -2.284                      | -2.393                      |
| 54020   | SLC37A1| Solute carrier family 37 (glycerol-3-phosphate transporter), member 1  | -2.112                      | -2.212                      |
| 2542    | SLC37A4| Solute carrier family 37 (glucose-6-phosphate transporter), member 4   | -2.117                      | -2.219                      |
| 151258  | SLC38A11| Solute carrier family 38, member 11                                    | 2.410                       | 3.085                       |
| 55089   | SLC38A4| Solute carrier family 38, member 4                                    | 1.837                       | 2.548                       |
| 92745   | SLC38A5| Solute carrier family 38, member 5                                    | -1.994                      | -2.152                      |
| 91252   | SLC39A13| Solute carrier family 39 (zinc transporter), member 13                 | 1.301                       | 2.070                       |
| 23516   | SLC39A14| Solute carrier family 39 (zinc transporter), member 14                | 2.569                       | 3.188                       |
| 29985   | SLC39A3| Solute carrier family 39 (zinc transporter), member 3                 | -2.032                      | -2.152                      |
| 283375  | SLC39A5| Solute carrier family 39 (metal ion transporter), member 5            | 1.623                       | 2.370                       |
| 7922    | SLC39A7| Solute carrier family 39 (zinc transporter), member 7                 | 1.273                       | 2.058                       |
| 30061   | SLC40A1| Solute carrier family 40 (iron-regulated transporter), member 1       | -3.612                      | -3.796                      |
| 84102   | SLC41A2| Solute carrier family 41, member 2                                    | 1.293                       | 2.070                       |
| 8501    | SLC43A1| Solute carrier family 43, member 1                                    | -2.013                      | -2.152                      |
| 57153   | SLC44A2| Solute carrier family 44, member 2                                    | -2.047                      | -2.152                      |
| 50651   | SLC45A1| Solute carrier family 45, member 1                                    | 2.038                       | 2.722                       |
| 146802  | SLC47A2| Solute carrier family 47, member 2                                    | -1.962                      | -2.026                      |
| 6521    | SLC4A1 | Solute carrier family 4, anion exchanger, member 1                    | -6.513                      | -6.453                      |
| 57282   | SLC4A10| Solute carrier family 4, sodium bicarbonate transporter, member 10   | -2.640                      | -2.753                      |
| 83959   | SLC4A11| Solute carrier family 4, sodium borate transporter, member 11         | 1.846                       | 2.569                       |
| 6508    | SLC4A3 | Solute carrier family 4, anion exchanger, member 3                    | 1.261                       | 2.045                       |
| 8671    | SLC4A4 | Solute carrier family 4, sodium bicarbonate cotransporter, member 4   | 2.445                       | 3.113                       |
| 9497    | SLC4A7 | Solute carrier family 4, sodium bicarbonate cotransporter, member 7   | 2.717                       | 3.307                       |
| 6523    | SLC5A1 | Solute carrier family 5 (sodium/glucose cotransporter), member 1       | -2.547                      | -2.705                      |
| 159963  | SLC5A12| Solute carrier family 5 (sodium/glucose cotransporter), member 12      | 4.753                       | 5.206                       |
| 6527    | SLC5A4 | Solute carrier family 5 (low affinity glucose cotransporter), member 4 | -3.969                      | -4.249                      |
| 6540    | SLC6A13| Solute carrier family 6 (neurotransmitter transporter, GABA), member 13| 1.328                       | 2.092                       |
| 5517    | SLC6A15| Solute carrier family 6 (neural amino acid transporter), member 15     | 1.623                       | 2.333                       |
| 388662  | SLC6A17| Solute carrier family 6, member 17                                    | 2.038                       | 2.728                       |
| 54716   | SLC6A20| Solute carrier family 6 (proline IMINO transporter), member 20         | 1.697                       | 2.433                       |
| 6532    | SLC6A4 | Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 | -2.512                      | -2.611                      |
| 6534    | SLC6A7 | Solute carrier family 6 (neurotransmitter transporter, L-proline), member 7 | 2.623                       | 3.228                       |
| 56301   | SLC7A10| Solute carrier family 7, (neutral amino acid transporter, y+ system), member 10 | -3.421                      | -3.603                      |
## Sarcoma

### (a) Continued.

| Gene ID | Symbol | Name | Tumor bone log2-fold change | Tumor muscle log2-fold change |
|---------|--------|------|-----------------------------|------------------------------|
| 6547    | SLC8A3 | Solute carrier family 8 (sodium/calcium exchanger), member 3 | −2.204 | −2.309 |
| 285335  | SLC9A10| Solute carrier family 9, member 10 | 1.208 | 2.000 |
| 6549    | SLC9A2 | Solute carrier family 9 (sodium/hydrogen exchanger), member 2 | 2.038 | 2.706 |
| 9368    | SLC9A3R1| Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1 | −2.760 | −2.846 |
| 9351    | SLC9A3R2| Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2 | 1.889 | 2.619 |
| 84679   | SLC9A7 | Solute carrier family 9 (sodium/hydrogen exchanger), member 7 | 3.347 | 3.935 |
| 10599   | SLC9A10| Solute carrier organic anion transporter family, member 1B1 | 3.360 | 3.977 |
| 28234   | SLC9A3R1| Solute carrier organic anion transporter family, member 3 regulator 1 | 9.760 | 9.569 |
| 6578    | SLC9A3R2| Solute carrier organic anion transporter family, member 3 regulator 2 | 2.432 | 3.096 |

### (b) Metabolism

| Gene ID | Symbol | Name | Tumor bone log2-fold change | Tumor muscle log2-fold change |
|---------|--------|------|-----------------------------|------------------------------|
| 1583    | CYP11A1| Cytochrome P450, family 11, subfamily A, polypeptide 1 | 1.623 | 2.396 |
| 1589    | CYP21A2| Cytochrome P450, family 21, subfamily A, polypeptide 2 | −1.962 | −2.036 |
| 1591    | CYP24A1| Cytochrome P450, family 24, subfamily A, polypeptide 1 | 5.208 | 5.577 |
| 1592    | CYP26A1| Cytochrome P450, family 26, subfamily A, polypeptide 1 | 2.623 | 3.257 |
| 1594    | CYP27B1| Cytochrome P450, family 27, subfamily B, polypeptide 1 | 1.846 | 2.585 |
| 339761  | CYP27C1| Cytochrome P450, family 27, subfamily C, polypeptide 1 | 3.585 | 4.178 |
| 1553    | CYP2A13| Cytochrome P450, family 2, subfamily A, polypeptide 13 | −1.962 | −2.035 |
| 1580    | CYP4B1 | Cytochrome P450, family 4, subfamily B, polypeptide 1 | −4.820 | −5.065 |
| 66002   | CYP4F12| Cytochrome P450, family 4, subfamily F, polypeptide 12 | −6.070 | −6.195 |
| 8529    | CYP4F2 | Cytochrome P450, family 4, subfamily F, polypeptide 2 | −7.769 | −7.060 |
| 4051    | CYP4F3 | Cytochrome P450, family 4, subfamily F, polypeptide 3 | −8.244 | −7.491 |
| 11283   | CYP4F8 | Cytochrome P450, family 4, subfamily F, polypeptide 8 | −5.006 | −5.270 |
| 260293  | CYP4X1| Cytochrome P450, family 4, subfamily X, polypeptide 1 | −2.476 | −2.580 |
| 9420    | CYP7B1 | Cytochrome P450, family 7, subfamily B, polypeptide 1 | 2.502 | 3.170 |
| 2326    | FMO1   | Flavin containing monoxygenase 1 | 4.360 | 4.859 |
| 2327    | FMO2   | Flavin containing monoxygenase 2 (nonfunctional) | −4.074 | −4.337 |
| 2328    | FMO3   | Flavin containing monoxygenase 3 | −4.036 | −4.309 |
| 388714  | FMO6P  | Flavin containing monoxygenase 6 pseudogene | −2.569 | −2.737 |
| 493869  | GPX8   | Glutathione peroxidase 8 (putative) | 2.814 | 3.371 |
| 2938    | GSTA1  | Glutathione S-transferase alpha 1 | 1.623 | 2.363 |
| 2939    | GSTA2  | Glutathione S-transferase alpha 2 | 2.038 | 2.741 |
| 2941    | GSTA4  | Glutathione S-transferase alpha 4 | 1.492 | 2.188 |
| 2953    | GSTT2  | Glutathione S-transferase theta 2 | 4.682 | 5.170 |
| 653689  | GSTT2B | Glutathione S-transferase theta 2B (gene/pseudogene) | 3.739 | 4.307 |
| 84779   | NAA11  | N(alpha)-acyltransferase 11, NaA catalytic subunit | 7.439 | 7.996 |
| 9027    | NAT8   | N-acetyltransferase 8 (GCN5-related, putative) | −2.769 | −2.920 |
| 7358    | UGDH   | UDP-glucose 6-dehydrogenase | 2.333 | 3.018 |
| 55757   | UGGT2  | UDP-glucose glycoprotein glucosyltransferase 2 | 1.604 | 2.271 |
| 10720   | UGT2B11| UDP glucuronosyltransferase 2 family, polypeptide B11 | −3.586 | −3.768 |
| 7367    | UGT2B17| UDP glucuronosyltransferase 2 family, polypeptide B17 | −2.836 | −2.959 |
| 54490   | UGT2B28| UDP glucuronosyltransferase 2 family, polypeptide B28 | −3.132 | −3.284 |
| 167127  | UGT3A2 | UDP glycosyltransferase 3 family, polypeptide A2 | −4.716 | −4.907 |
Table 4: FAF1 mutations in various cancers. FAF1 mutations listed in the TCGA data base were identified without restriction to any type of cancer. For the location of the affected domains on the protein compare Figure 2.

| AA    | Mutation | Cancer                                | Domain  |
|-------|----------|---------------------------------------|---------|
| N4S   | Missense | Cutaneous melanoma                     |         |
| I10S  | Missense | Stomach adenocarcinoma                 |         |
| E21   | Nonsense | Cutaneous melanoma                     | UBA     |
| E25K  | Missense | Uterine endometrioid carcinoma         | UBA     |
| V38   | Splice   | Stomach adenocarcinoma                 | UBA     |
| P86fs | FS del   | Colorectal adenocarcinoma              |         |
| G123  | Splice   | Uterine endometrioid carcinoma         | UB1     |
| P136H | Missense | Colorectal adenocarcinoma              | UB1     |
| T147M | Missense | Brain lower grade glioma               | UB1     |
| D149Y | Missense | Uterine endometrioid carcinoma         | UB1     |
| L159V | Missense | Lung adenocarcinoma                    | UB1     |
| K163N | Missense | Uterine endometrioid carcinoma         | UB1     |
| L170F | Missense | Cutaneous melanoma                     |         |
| G184  | Splice   | Colorectal cancer                      |         |
| Q187H | Missense | Stomach adenocarcinoma                 |         |
| S214N | Missense | Colorectal adenocarcinoma              | UB2     |
| R222I | Missense | Uterine endometrioid carcinoma         | UB2     |
| E238D | Missense | Lung adenocarcinoma                    | UB2     |
| P241S | Missense | Uterine endometrioid carcinoma         | UB2     |
| T245A | Missense | Renal clear cell carcinoma             | UB2     |
| M249V | Missense | Uterine endometrioid carcinoma         | UB2     |
| E280K | Missense | Brain lower grade glioma               | UB2     |
| G293  | Nonsense | Colorectal cancer                      |         |
| T300I | Missense | Colorectal adenocarcinoma              |         |
| D305H | Missense | Lung adenocarcinoma                    |         |
| E308Q | Missense | Lung adenocarcinoma                    |         |
| A316V | Missense | Stomach adenocarcinoma                 |         |
| K319fs| FS ins   | Head and neck squamous cell carcinoma  |         |
| R344G | Missense | Stomach adenocarcinoma                 |         |
| F355I | Missense | Cutaneous melanoma                     | UAS     |
| L379V | Missense | Breast invasive carcinoma              | UAS     |
| C396F | Missense | Cutaneous melanoma                     | UAS     |
| S459  | Nonsense | Uterine endometrioid carcinoma         | UAS     |
| G469  | Splice   | Glioblastoma multiforme                | UAS     |
| R509G | Missense | Lung squamous cell carcinoma           |         |
| E510fs| FS del   | Cutaneous melanoma                     |         |
| R516C | Missense | Uterine endometrioid carcinoma         |         |
| A534V | Missense | Stomach adenocarcinoma                 |         |
| F537L | Missense | Stomach adenocarcinoma                 |         |
| E551  | Nonsense | Lung adenocarcinoma                    |         |
| R554W | Missense | Colorectal cancer                      |         |
| S582I | Missense | Lung adenocarcinoma                    |         |
| F585L | Missense | Uterine endometrioid carcinoma         | UBX     |
| E587  | Nonsense | Lung adenocarcinoma                    | UBX     |
| A592V | Missense | Stomach adenocarcinoma                 | UBX     |
| W610  | Nonsense | Breast invasive carcinoma              | UBX     |
| D611Y | Missense | Lung adenocarcinoma                    | UBX     |
| E635fs| FS del   | Brain lower grade glioma               | UBX     |
| P640fs| FS del   | Cutaneous melanoma                     | UBX     |
a more accurate reference point for a leiomyosarcoma would have been smooth muscle, we believe that the comparison to striated muscle is sufficient to allow the assessment of tumor specific changes. We have measured RNA, DNA, and protein with various assays. Similar assessments in the future should also include chromosome analysis for possible translocations.

In the first-line defense against cancer, the gradual replacement of conventional chemotherapy with molecularly targeted agents has opened the possibility to tailor drug treatments to particular tumors. This transition necessitates the characterization of the molecular lesions that are causative for the transformation of healthy cells into cancerous cells, because drugs need to be matched with the underlying carcinogenic defect to be effective. An additional caveat, caused by unique genetic changes in the primary tumor, can affect drug transport and metabolism and needs to be taken into consideration. In this case, the RNA levels for genes that regulate transport and metabolism were extensively skewed in the tumor tissue as compared to muscle and bone (see Table 3), implying potential challenges to chemotherapy. An advanced molecular treatment strategy for cancer will rely on the molecular definition of drug target, drug transport, and drug metabolism pharmacogenetics in the primary tumor. Consecutive to cancer dissemination, it will also require adjustments to account for the genetic changes in the metastases.

The cost of health care has been under increasing scrutiny. The treatment of cancer patients is expensive, in particular when hospitalization is required, and further in cases of end-of-life care. Avoidable expenditures are generated by suboptimal treatment decisions that result in low efficacy or hightoxicityofanticancerregimens. Molecularmedicinehas impliedpotentialtopreemptthoseproblemsandreducewasteful use of resources. The potential for preempting those problems and reduce wasteful spending. Yet, it requires the upfront cost of molecular cancer examination. The analysis performed in this study required about $11,000 in nonsalary expenses to perform. While it has not identified a drug target, it has specified possible confines for drug treatment. The costs for molecular analysis need to be weighed against the societal cost derived from lost productivity in the workforce, disrupted lives of families, and premature deaths. While currently limited drug options constitute the major constraint to the approach taken here, the foreseeable future will bring an increasing spectrum of molecularly targeted drugs along with faster and cheaper technologies for the molecular assessment of cancers. They will facilitate the clinical translation of our approach.

Consent

The patient provided informed consent.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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