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# *ESR1* hotspot mutations in endometrial stromal sarcoma with high-grade transformation and endocrine treatment

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High-grade endometrial stromal sarcomas (HGESSs) are more aggressive and have higher rates of resistance to endocrine therapy than low-grade endometrial stromal sarcomas (LGESSs). The pathogenesis of hormonal resistance in these lesions has yet to be defined. Here we sought to histologically and genetically characterize 3 LGESSs and their recurrences that underwent histologic high-grade transformation following endocrine therapy. For this, DNA from primary tumors and select subsequent recurrences were subject to massively parallel sequencing targeting 468 cancer-related genes. Somatic mutation analyses were performed using validated bioinformatics methods. In addition, RNA from each case was evaluated for the presence of gene fusions using targeted RNA-sequencing. All patients initially presented with LGESS, developed HGESS recurrences, and received at least 2 lines of hormonal suppressive therapy. Gene fusions classically described as associated with LGESS were identified in all 3 cases, including *JAZF1-PHF1*, *EPC1-PHF1* and *JAZF1-SUZ12* fusions for Cases 1, 2 and 3, respectively. Targeted sequencing analysis revealed that none of the primary LGESS, however the HGESS recurrences of Cases 1 and 3, and the LGESS and HGESS recurrences of Case 2 post endocrine treatment harbored *ESR1* p.Y537S hotspot mutations. These *ESR1* ligand-binding domain mutations have been found as a mechanism of acquired endocrine resistance in breast cancer. Also, a reduction in estrogen receptor (ER) expression was observed in recurrences. Our findings suggest that the *ESR1* p.Y537S hotspot mutation in LGESS with histologic high-grade transformation may be associated with endocrine resistance in these lesions. Furthermore, our data suggest that genetic analyses may be performed in recurrent LGESS following hormonal therapy, development of high-grade morphology, and/or altered/diminished ER expression.

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## INTRODUCTION

Endometrial stromal sarcomas (ESSs) are rare, accounting for ~15% of uterine sarcomas, and are classified as low- (LGESS) or high-grade (HGESS) based on histologic, immunophenotypic, and molecular genetic features[1]. LGESS are characterized by spindle cells with mild nuclear atypia, low mitotic activity, CD10, estrogen receptor (ER), progesterone receptor (PR) expression, and recurrent chromosomal rearrangements often involving *JAZF1*[2–4]. HGESS consist of round and/or spindle cells demonstrating moderate nuclear atypia, brisk mitotic activity, and loss of CD10 and/or ER and PR expression with cyclin D1 and BCOR overexpression[1]. Most HGESS harbor *YWHAE* and *BCOR* fusions or *BCOR* internal tandem duplications[3,4]. Rare tumors harbor LGESS-associated gene fusions and demonstrate increased nuclear atypia and mitotic index warranting classification as HGESS[5].

Prognostication of ESS, particularly high-grade variants, is difficult due to the evolving uterine sarcoma classification with increased utility of massively parallel sequencing. LGESS are indolent tumors treated with surgery, hormonal blockade, or a combination thereof[6,7]. While LGESS has a 5-year disease-free survival rate of >90%, up to 60% of patients develop late recurrences[8–10]. Some LGESS patients develop resistance to hormonal treatment and may benefit from cytotoxic chemotherapy[6,11,12]. HGESS, including histologically transformed ESS with LGESS-associated fusions, appear to have a more aggressive behavior compared to LGESS and is treated by surgery and chemotherapy with improved response using anthracycline-based regimens in tumors harboring *YWHAE* fusions[13]. Mechanisms of hormonal resistance in LGESS have not been previously explored, and the pathogenesis of histologically transformed LGESS is unknown. Here we sought to histologically and genetically characterize 3 LGESS and their recurrences that underwent histologic high-grade transformation following endocrine therapy.

## METHODS

### Cases

The patients provided informed consent for a Memorial Sloan Kettering Cancer Center (MSK) Institutional Review Board-approved tumor genomic

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profiling study. Between 2014 and 2018, 18 diagnostically confirmed LGESS and HGESS were subjected to clinical sequencing at our institution. Three cases with *ESR1* mutations and available tissues were identified. Formalin-fixed paraffin-embedded (FFPE) tissues of primary tumors and matched recurrences were obtained. All available hematoxylin and eosin and ER, PR, BCOR, and cyclin D1 immunohistochemical stained slides were reviewed by expert pathologists (S.C., R.A.S.). Cases were histologically subtyped using the World Health Organization classification system[14]. ER and PR expression were interpreted as positive if ≥1% of tumor cells showed nuclear staining, and intensity of staining was recorded[2]. ER and PR Allred scores were calculated as the sum of proportion and intensity scores. The proportion score was assigned as 0 (0%), 1 (<1%), 2 (1–10%), 3 (11–33%), 4 (34–66%), and 5 (67–100%). The intensity score was assigned as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong)[15]. BCOR and cyclin D1 were interpreted as positive if ≥50% of tumor cells showed nuclear staining[3,4]. Clinical data, including demographics, treatment, and follow-up, were extracted from the electronic medical record.

### Targeted massively parallel sequencing and validation

Representative 8 µm-thick sections from representative LGESS and HGESS FFPE tumor blocks were subjected to microdissection using a sterile needle under a stereomicroscope when appropriate to ensure >80% tumor cell content, as previously described[16,17]. Genomic DNA was extracted from the tumor samples and matched normal blood or normal tissue devoid of neoplastic cells using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturers' instructions[18]. Tumor and matched normal DNA samples were subjected to massively parallel sequencing targeting 468 genes using MSK-Integrated Mutational Profiling of Actionable Cancer Targets (MSK-IMPACT)[16,19] at a median depth of 595x (range, 584-625x), and sequencing data were analyzed using validated bioinformatics tools as previously described[16,17]. In brief, somatic single nucleotide variants (SNVs) were identified using MuTect (v1.17)[20], and small insertions and deletions (indels) using Strelka (v1.0.15), VarScan 2 (v2.3.7), Lancet (v1.0.0) and Scalpel (v0.5.3)[21–24]. Mutations identified in the primary LGESS or recurrence from a given patient were interrogated in the respective LGESS and/or recurrence(s) by manual inspection of BAM files using mpileup files (SAMtools version 1.2 htslib 1.2.1)[25]. Copy number alterations (CNAs) and loss of heterozygosity (LOH) were defined using FACETS[26], as previously described[16,17]. Cancer cell fractions (CCFs) of somatic mutations identified were defined using ABSOLUTE (v1.0.6)[27], and a mutation was classified as clonal if its probability of being clonal was >50% or if the lower bound of the 95% confidence interval of its CCF was >90%, as previously described[16,17]. Mutational hotspot annotation was performed according to Chang et al[28].

*ESR1* p.Y537S mutations identified by MSK-IMPACT sequencing were subjected to orthogonal validation using Sanger sequencing, as previously described[29]. In brief, PCR amplification was performed using the AmpliTaq Gold 360 Master Mix kit (Life Technologies, ThermoFisher Scientific; primers: forward 5′-CCTTTCTGTGTCTTCCCACCT, reverse 3′-AGTGGCTTT GGTCCGTCTC), and PCR fragments were cleaned using ExoSAP-IT (ThermoFisher Scientific).

### Targeted fusion gene detection

Tumor samples were subjected to the MSK-Fusion Solid Panel assay (Archer FusionPlex) utilizing Archer Anchored Multiplex PCR technology to detect 85 validated gene fusions in solid tumors, as previously described[5]. For this, tumor RNA was extracted from 5-µm-thick tumor sections, followed by cDNA synthesis and library preparation. Final targeted amplicons were sequenced, and data analyzed using the Archer analysis software V5.0.

## RESULTS

### Case 1

Case 1 was a 47-year-old woman who, after resection, was found to have FIGO stage IIIB LGESS with residual miliary disease (Fig. 1A). The primary uterine (Case1-Prim) and extrauterine tumor demonstrated predominately conventional LGESS morphology characterized by small monomorphic spindle cells with round to oval nuclei, inconspicuous nucleoli, scant cytoplasm, and a mitotic index of <1 per 10 high power fields (HPF; Fig. 1B); focal variant decidual change was present. Strong ER and PR expression was observed in >90% of tumor cells (ER and PR Allred scores 8 and 8, respectively; Fig. 1B). She received post-resection megestrol acetate and remained radiographically disease-free for 6.5 years. A large recurrent jejunal mass was partially resected in the setting of a small bowel obstruction (Case1-Recur1), and she received post-resection anastrozole for 3.5 years, at which time attempted resection of persistent disease was not successful (Case1-Recur2). Treatment was changed to letrozole with disease control for 2 years, after which the patient had successful complete resection of the intra-colonic mass (Case1-Recur3) and peritoneal disease.

All recurrences (Case1-Recur1/Recur2/Recur3) demonstrated LGESS morphology with foci of high-grade histologic transformation characterized by nuclear enlargement, prominent nucleoli, and increased mitotic activity of 9–11 mitoses per 10 HPF (Fig. 1B). The first and second recurrences (Case1-Recur1/Recur2) demonstrated ER and PR expression with variable intensity in >90% of tumor cells, including histologically high-grade foci (ER and PR Allred scores 7 and 7 in Case1-Recur1, respectively; ER and PR Allred scores 8 and 7 in Case1-Recur2, respectively). The third recurrence (Case1-Recur3) demonstrated strong PR but weak ER expression in 90% of tumor cells (ER and PR Allred scores 6 and 8, respectively). Cyclin D1 and BCOR were negative in all recurrences (Case1-Recur1/Recur2/Recur3).

A *JAZF1-PHF1* fusion was detected. Targeted massively parallel sequencing revealed the presence of a somatic *KDM5C* frameshift mutation in the primary and second and third recurrent tumors (Case1-Prim/Recur2/Recur3; Fig. 1C); the first recurrence (Case1-Recur1) had insufficient tissue for sequencing. Notably, the third recurrence (Case1-Recur3) acquired a *STK40* missense p.R128W mutation and an *ESR1* p.Y537S hotspot mutation (variant allele fraction (VAF), 40%) not present in the primary tumor or second recurrence (Case1-Prim/Recur2; Fig. 1C). We next defined the cancer cell fraction (CCF; i.e., the bioinformatically inferred percentage of tumor cells harboring the mutation in the sample) of the *ESR1* mutation, taking into account the tumor purity, ploidy and local copy number[27], which revealed that the *ESR1* p.Y537S hotspot mutation was clonal (i.e., present in 100% of the cancer cells; Fig. 1C). With the finding of an *ESR1* mutation in the third recurrence (Case1-Recur3), the patient was treated with fulvestrant and continues to have no measurable disease for 48 months, 15 years from initial diagnosis (Fig. 1A).

### Case 2

A 56-year-old woman was found to have FIGO stage IB LGESS (Fig. 2A), with a mass resected from the vaginal cuff (Case2-Prim). The patient was treated with post-resection letrozole and remained disease-free for 23 months, at which time imaging showed recurrent pelvic disease. Megestrol acetate was added to the letrozole, but there was rapid progression of disease. She was subsequently treated with gemcitabine-docetaxel, but the disease continued to progress. She underwent resection of multi-site peritoneal and retroperitoneal disease (Case2-Recur1) followed by observation for 11 months. For subsequent progression, she underwent another resection (Case2-Recur2), and then progressed 4 months later. She was treated with doxorubicin but had progression of disease.

The primary tumor and first recurrence (Case2-Prim/Recur1) showed conventional and variant sex cord-like LGESS morphology and a mitotic index of 2/10 HPF (Fig. 2B). PR expression was strong in >90% of tumor cells in the primary tumor and first recurrence (Case2-Prim/Recur1; PR Allred scores of 8 and 8, respectively); however, ER expression was strong in >90% of tumor cells in the primary tumor (ER Allred score 8; Case2-Prim) and weak in 10% of tumor cells of the first recurrence (ER Allred score 3; Case2-Recur1; Fig. 2B). The second recurrence (Case2-Recur2) involved multiple peritoneal and retroperitoneal sites mostly demonstrating conventional and variant sex cord-like LGESS morphology with moderate ER expression in 60% of tumor cells and strong PR expression in >90% of tumor cells (ER and PR Allred scores of 6

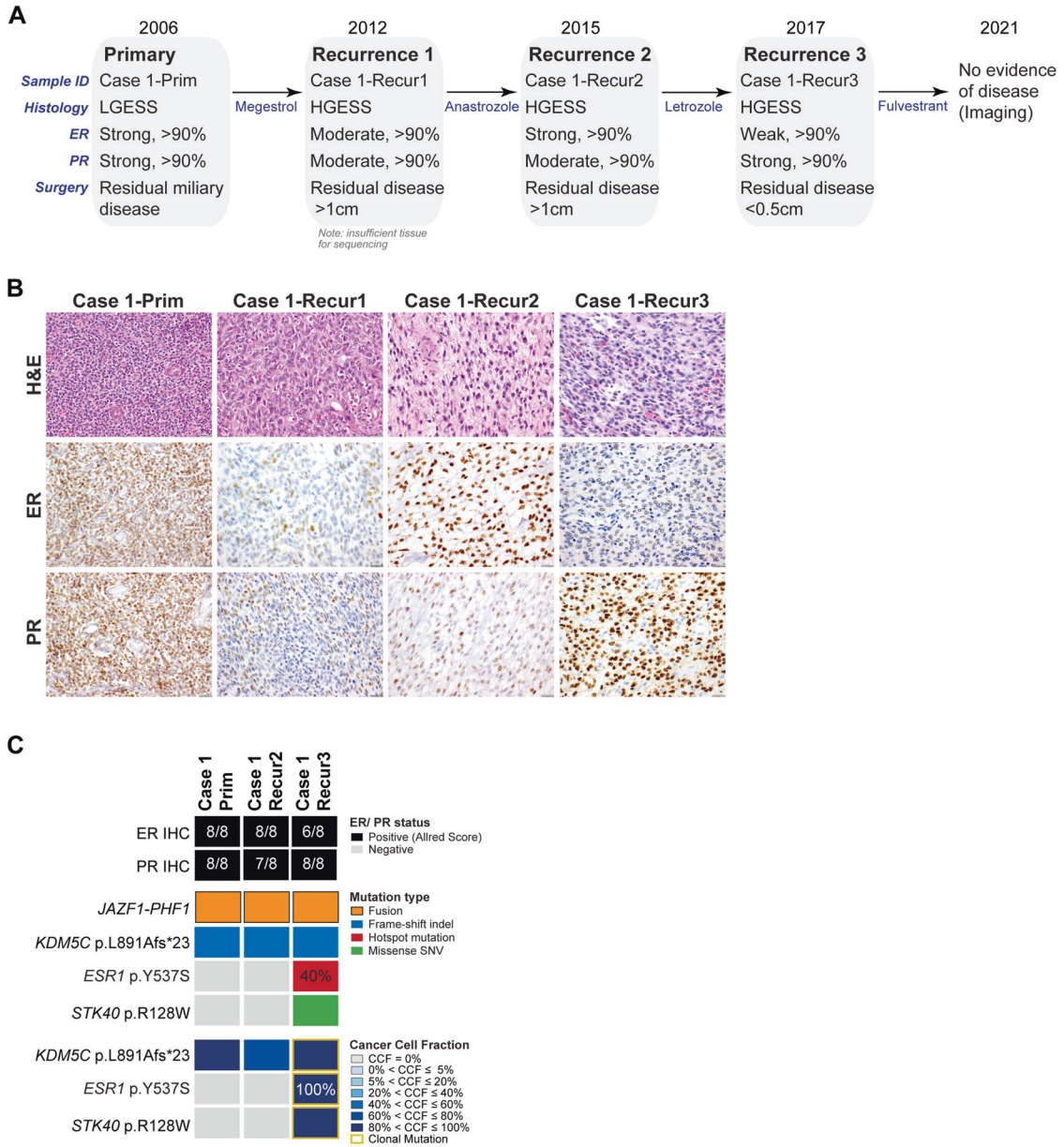


**Fig. 1 Histologic and genomic analysis of Case 1. A** Clinical, surgical and treatment history including histology and estrogen receptor (ER)/ progesterone receptor (PR) status of the primary low-grade endometrial stromal sarcoma (LGESS) (Case1-Prim) and recurrences (Case1-Recur1/Recur2/Recur3). **B** Micrographs of representative hematoxylin and eosin (H&E)-stained sections and ER/PR expression by immunohistochemistry of the primary LGESS (Case1-Prim) and each recurrence (Case1-Recur1/Recur2/Recur3). Magnification ×20. **C** Fusion gene and non-synonymous somatic mutations identified in the primary LGESS (Case1-Prim) and the second (Case1-Recur2) and third (Case1-Recur3) recurrences. Mutation types (middle), including the variant allele fraction for the *ESR1* mutation, and cancer cell fraction of mutations identified (bottom) are color-coded according to the legend. Note the clonal *ESR1* hotspot mutation was only detected in the last recurrence (Case1-Recur3). The Allred scores for ER and PR are provided in the phenobar. LGESS, low-grade endometrial stromal sarcoma; HGESS, high-grade endometrial stromal sarcoma.

and 8, respectively). The para-aortic recurrence (Case2-Recur2), however, demonstrated round cells with enlarged nuclei, abnormal chromatin, and 12 mitoses per 10 HPF consistent with histologic high-grade transformation (Fig. 2B). These foci demonstrated strong ER/PR expression in >90% of tumor cells (ER and PR Allred scores of 8 and 8, respectively; Fig. 2B) and were positive for BCOR, while negative for cyclin D1.

An *EPC1-PHF1* fusion was detected. Massively parallel sequencing revealed the presence of an *ESR1* p.Y537S hotspot mutation in the recurrences (Case2-Recur1, VAF 43%; Case2-Recur2, VAF 29%) but not in the primary tumor (Case2-Prim; Fig. 2C). Analysis to define the CCFs showed that the *ESR1* hotspot mutation was clonal and present in 100% of the cancer cells of both recurrences. Noting the *ESR1* mutation on the most recent pathology (Case2-Recur2), the patient was treated with fulvestrant with radiographic response sustained for 1 year. At further progression, she was changed to anastrozole, then deceased 6 months later, 6 years from initial diagnosis.

### Case 3

A 56-year-old was found to have FIGO stage IIIB LGESS after resection (Case3-Prim; Fig. 3A). She was treated with post-resection megestrol acetate for 11 years. Over 8 years since her initial surgery, she underwent resection of an intrabdominal mass,

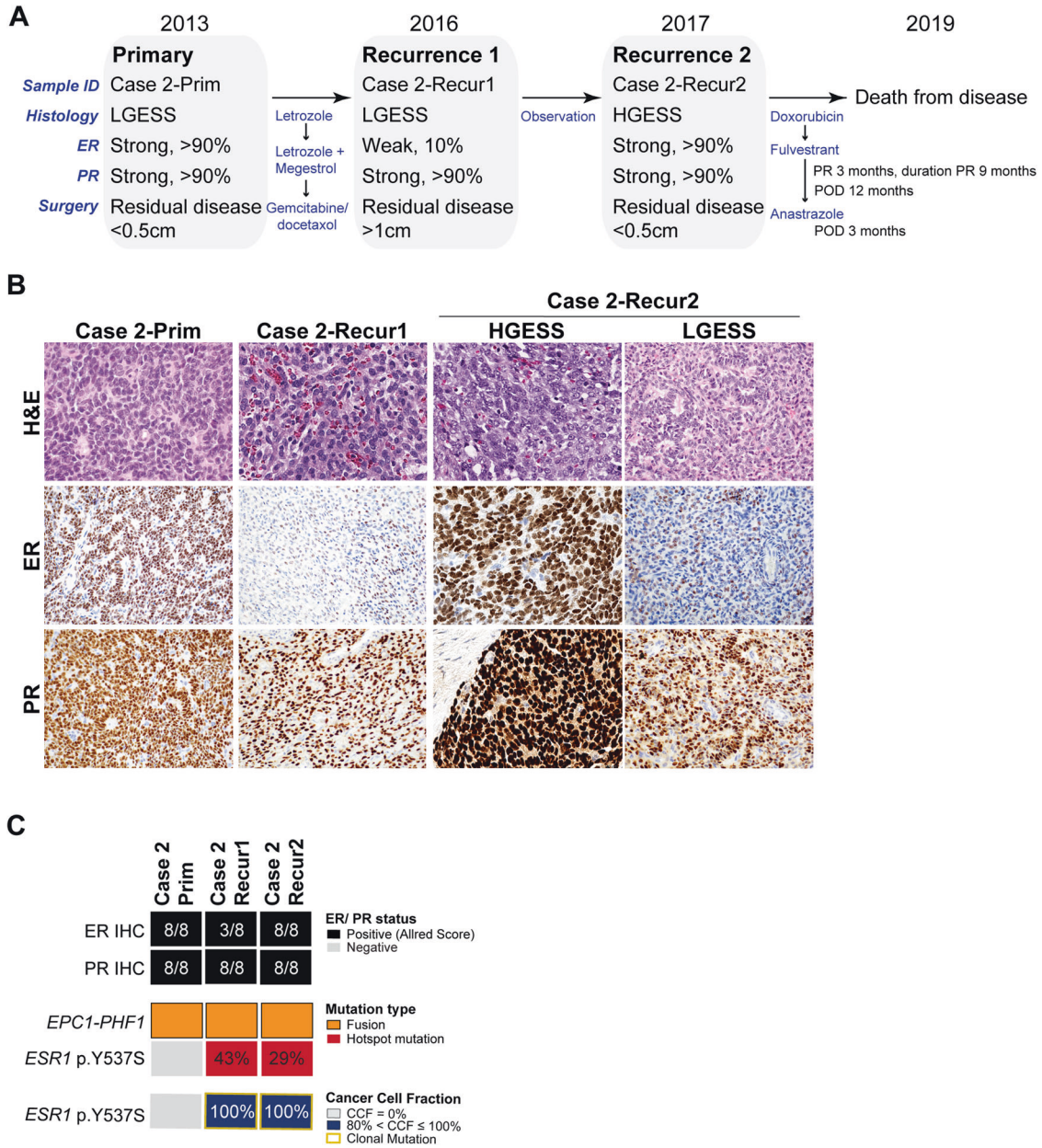


**Fig. 2 Histologic and genomic analysis of Case 2. A** Clinical, surgical and treatment history including histology and estrogen receptor (ER)/ progesterone receptor (PR) status of the primary low-grade endometrial stromal sarcoma (LGESS) (Case2-Prim) and recurrences (Case2-Recur1/Recur2). **B** Micrographs of representative hematoxylin and eosin (H&E)-stained sections and ER/PR expression by immunohistochemistry of the primary LGESS (Case2-Prim) and each recurrence (Case2-Recur1/Recur2). For Case2-Recur2, the low-grade component of the HGESS was subjected to ER and PR immunohistochemical analysis separately. Magnification ×20. **C** Fusion gene and non-synonymous somatic mutations identified in the primary LGESS (Case2-Prim) and first (Case2-Recur1) and second (Case2-Recur2) recurrences. Mutation types (middle), including the variant allele fraction for the *ESR1* mutation, and cancer cell fraction of mutations identified (bottom) are color-coded according to the legend. Note the clonal *ESR1* hotspot mutation was identified in both recurrences (Case2-Recur1/Recur2). The Allred scores for ER and PR are provided in the phenobar. LGESS low-grade endometrial stromal sarcoma, HGESS high-grade endometrial stromal sarcoma, POD progression of disease, PR partial response.

followed by splenectomy and a small bowel resection. While no recurrent disease was found in the splenectomy specimen, the pathology from the other 2 surgeries were not reviewed. She presented to our institution 11 years after initial diagnosis with 2 large recurrent abdominal masses, which were debulked (Case3-Recur1), and she remained disease-free while on post-resection letrozole for 6 years. She underwent surgery, including removal of an infected mesh and small bowel partial resection, with no evidence of recurrent disease intraoperatively. Letrozole was resumed, but she was found to have peritoneal nodules on imaging a year later, and underwent resection for recurrent disease (Case3-Recur2).

The primary tumor demonstrated LGESS morphology and a mitotic index of <1/10 HPF; strong ER and PR staining was seen in >90% of tumor cells (ER and PR Allred scores of 8 and 8, respectively; Fig. 3B). The first recurrence (Case3-Recur1) demonstrated epithelioid and spindled cells with enlarged nuclei with moderate to severe atypia, abundant eosinophilic cytoplasm, prominent myxoid change, and <1 mitotic figure per 10 HPF, associated with a prominent lymphocytic infiltrate; PR expression

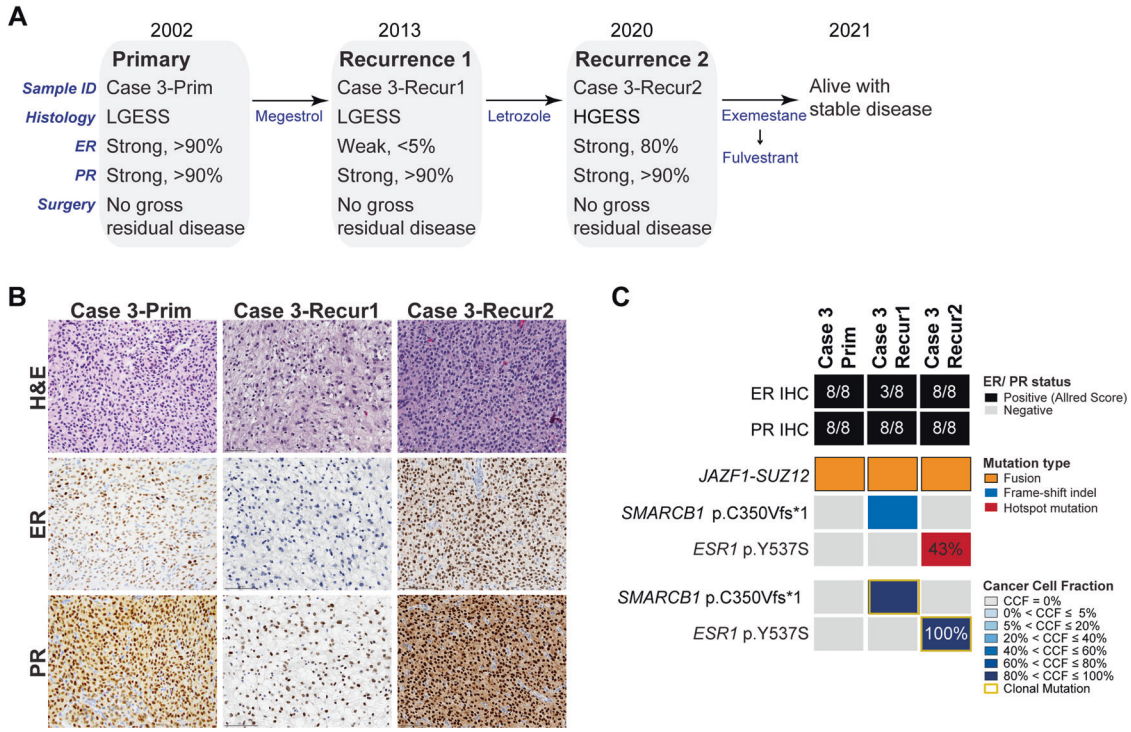


**Fig. 3 Histologic and genomic analysis of Case 3. A** Clinical, surgical and treatment history including histology and estrogen receptor (ER)/ progesterone receptor (PR) status of the primary low-grade endometrial stromal sarcoma (LGESS) (Case3-Prim) and recurrences (Case3-Recur1/Recur2). **B** Micrographs of representative hematoxylin and eosin (H&E)-stained sections and ER/PR expression by immunohistochemistry of the primary LGESS (Case3-Prim) and each recurrence (Case3-Recur1/Recur2). Magnification ×20. **C** Fusion gene and non-synonymous somatic mutations identified in the primary LGESS (Case3-Prim) and first (Case3-Recur1) and second (Case3-Recur2) recurrences. Mutation types (middle), including the variant allele fraction for the *ESR1* mutation, and cancer cell fraction of mutations identified (bottom) are color-coded according to the legend. Note the clonal *ESR1* hotspot mutation was identified only the second recurrence (Case3-Recur2). The Allred scores for ER and PR are provided in the phenobar. LGESS, low-grade endometrial stromal sarcoma; HGESS, high-grade endometrial stromal sarcoma.

was strong in >90% of tumor cells, while ER was weak in <5% of cells in the first recurrence (PR and ER Allred scores of 8 and 3, respectively; Case3-Recur1; Fig. 3B). The second recurrence (Case3-Recur2) demonstrated spindle cells with uniform moderate cytologic atypia, including nuclear enlargement and prominent nucleoli, and a mitotic index of 10/10 HPF, consistent with high-grade transformation; no epithelioid or myxoid change was present (Fig. 3B). ER and PR expression was strong in 80% and >90% of cells, respectively (ER and PR Allred scores of 8 and 8, respectively).

A *JAZF1-SUZ12* fusion was detected. Massively parallel sequencing revealed the presence of a frameshift mutation in *SMARCB1* in the first recurrence (Case3-Recur1) and a clonal *ESR1* p.Y537S hotspot mutation in the second recurrence (Case3-Recur2, VAF 43%; Fig. 3C). Akin to Case 1 and Case 2, the *ESR1* hotspot mutation was clonal in the second recurrence (Case3-Recur2), with all cancer cells harboring this mutation (CCF 100%; Fig. 3C). Noting the *ESR1* mutation on the most recent pathology (Case3-Recur2), the patient completed 3 cycles of fulvestrant and is alive with disease, 19 years from initial diagnosis.

## DISCUSSION

Here we report on likely de novo *ESR1* p.Y537S hotspot mutations in the recurrences of 3 patients who were initially diagnosed with LGESS and subsequently developed recurrent disease demonstrating histologic high-grade transformation after hormonal treatment.

The *ESR1* gene codes for the estrogen receptor 1 (ERalpha), a ligand-activated transcription factor implicated in the tumorigenesis of many ER-positive cancers including ESS[30,31]. *ESR1* p.Y537S hotspot mutations are activating and enable ER coactivator binding in the absence of estrogen[32,33]. Mutations in the *ESR1* ligand-binding domain have been identified as an important mechanism of acquired endocrine resistance and occur under the selective pressure from hormonal therapies[34,35]. Particularly in breast cancer, *ESR1* mutations have been found in ~1% of primary ER-positive breast cancers and 10–50% of metastatic breast cancers previously treated with hormonal blockade[34,36,37]. Also, a de novo *ESR1* hotspot mutation in a patient with endometrial carcinoma treated with an aromatase inhibitor has been reported[38].

Other mechanisms of endocrine resistance include *ESR1* amplification/ fusion and alterations in receptor tyrosine kinases, PI3K and MAPK pathway components, regulators of gene expression, and DNA repair genes. We explored these alterations in the remaining 15 ESS of the 18 diagnostically confirmed ESS subjected to clinical sequencing at our institution (see Methods). Somatic genetic alterations associated with endocrine resistance were detected in only 2 cases: a recurrent HGESS harboring a *YWHAE* rearrangement showed *TSC2* and *MTOR* deep deletions as well as *AKT2* amplification. *AKT1* amplification and *PMS2* deletion were detected in a primary LGESS with a *JAZF1-SUZ12* fusion. Among the 15 ESS with sequencing data, 3 were histologically confirmed LGESS lacking *ESR1* mutations, and none of these 3 patients had evidence of hormonal resistance.

The association between ER expression and *ESR1* mutation in our cohort is unclear. In Cases 1 and 2, ER expression was significantly attenuated in the recurrences that harbored *ESR1* mutations. In Case 3, however, ER expression was diminished in the first recurrence with wild-type *ESR1*. Interestingly, in Cases 2 and 3, the recurrences that initially showed reduced ER

expression were followed by recurrences with strong levels of ER expression.

In all cases studied here, histologically high-grade transformation of LGESS was observed during disease progression as evidenced by increased mitotic rates and nuclear atypia in the recurrences, which paralleled the detection of *ESR1* p.Y537S hotspot mutations. While Case 2 displayed endocrine resistance at first recurrence, in Cases 1 and 3 prolonged disease control was observed on a selective ER degrader (SERD; fulvestrant) following resistance to a selective ER modulator (SERM)/aromatase inhibitor (AI). In fact, work in breast cancer has shown that the mechanism of resistance to SERMs/AIs is distinct from that of SERDs[39,40]. All 3 patients had some degree of clinical benefit from fulvestrant, which was administered after detection of *ESR1* hotspot mutations in the recurrences. Further studies are warranted to assess whether patients with *ESR1*-mutant ESS may benefit from SERDs over other therapies.

High-grade transformation of LGESS is rare, and data on the molecular underpinnings of these lesions are limited. The majority of LGESS are driven by *PHF1* and *JAZF1* translocations[5,41], as the 3 cases described in this study, whereas *YWHAE* and *BCOR* genetic abnormalities are associated with HGESS[42]. In Case 1, this occurred prior to the identification of the *ESR1* mutation and in Cases 2 and 3, the high-grade transformation was identified after the emergence of an *ESR1* mutation. Notably in Case 3, however, there was increased nuclear atypia despite low mitotic activity in the first recurrence (Case3-Recur1), suggesting early transformation at that time. The interplay between ER-alpha signaling and histologic high-grade transformation of LGESS remains unknown and warrants further investigation.

Ligand independent activation of ER receptors results in increased translational activity outside of the downstream affects typically attributed to estrogen binding[43,44]. For example, *ESR1* mutations were found to result in increased expression of non-ER regulation genes associated with breast cancer cell migration such as *WNT11*[45] and *RET*[46], suggesting that these mutations may contribute to increased metastatic potential of these tumors[33]. This theory is supported by identification of these mutations preferentially in distant metastatic sites in comparison to locoregional recurrences[37,47]. Thus, the presence of an *ESR1* mutation may have prognostic implications separate from the limitation on effective therapies.

This study has several limitations. Given the rarity of LGESS in general, and of those undergoing high-grade transformation in particular, the number of cases studied is small, however the identification of potential mechanisms of endocrine resistance contributes to the body of knowledge in this disease. In addition, ER and PR immunohistochemical analyses were performed at the referring institution (Case 1) or at our clinical and research laboratories (Cases 1–3), which may contribute to differences in staining intensities.

In summary, de novo *ESR1* hotspot mutations may occur in LGESS following histologic high-grade transformation and resistance to endocrine treatment. Larger series are required to further investigate the frequency of *ESR1* mutations and their role in endocrine treatment resistance. Our findings suggest that genetic analyses may be performed in recurrent LGESS following hormonal therapy, development of high-grade morphology, and/or altered/ diminished ER expression.

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Conception and design: M.L.H., B.W., S.C. Financial support: N.R.A.-R. Provision of study material or patients: S.C. Collection and assembly of data: K.D., K.M.M., E.K., A.D.C.P., Y.Z., E.M.d.S., S.D., R.B., C.W.A., R.A.S., M.L.H., B.W., S.C. Data analysis and interpretation: K.D., K.M.M., E.K., A.D.C.P., P.S., B.W., S.C. Manuscript writing: K.D., K.M.M., E.K., M.L.H., B.W., S.C. Final approval of manuscript: All authors.

## COMPETING INTERESTS

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