Constitutively active *ESR1* mutations in gynecologic malignancies and clinical response to estrogen-receptor directed therapies

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HIGHLIGHTS

- *ESR1* mutations lead to constitutive activation of ERα and resistance to aromatase inhibitors.
- A subset of gynecologic malignancies, particularly those with endometrioid histology, harbor *ESR1* mutations.
- *ESR1* mutations may arise in the setting of treatment with AIs, or may be present in the initial tumor.
- SERM/SERD therapy has the potential to provide benefit despite the presence of an *ESR1* mutation.

ARTICLE INFO

ABSTRACT

Objective. Endocrine therapy is often considered as a treatment for hormone-responsive gynecologic malignancies. In breast cancer, activating mutations in the estrogen receptor (*mutESR1*) contribute to therapeutic resistance to endocrine therapy, especially aromatase inhibitors (AIs). The purpose of this study was to evaluate the frequency and clinical relevance of *ESR1* genomic alterations in gynecologic malignancies.

Methods. DNA from FFPE tumor tissue obtained during routine clinical care for 9645 gynecologic malignancies (ovary, fallopian tube, uterus, cervix, vagina, vulvar, and placenta) was analyzed for all classes of genomic alterations (base substitutions (muts), insertions, deletions, rearrangements, and amplifications) in *ESR1* by hybrid capture next generation sequencing. A subset of alterations was characterized in laboratory-based transcription assays for response to endocrine therapies.

Results. A total of 295 *ESR1* genomic alterations were identified in 285 (3.0%) cases. *mutESR1* were present in 86 (0.9%) cases and were more common in uterine compared to other cancers (2.0% vs <1%, respectively *p* < 0.001). *mutESR1* were enriched in carcinomas with endometrioid versus serous histology (4.4% vs 0.2% respectively, *p* < 0.0001 in uterine and 3.5% vs 0.3% respectively, *p* = 0.0004 in ovarian carcinomas). In three of four patients with serial sampling, *mutESR1* emerged under the selective pressure of AI therapy. Despite decreased potency of estrogen receptor (ER) antagonists in transcriptional assays, clinical benefit was observed following treatment with selective ER-targeted therapy, in one case lasting >48 months.

Conclusions. While the prevalence of *ESR1* mutations in gynecologic malignancies is low, there are significant clinical implications useful in guiding therapeutic approaches for these cancers.

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1. Introduction

Gynecologic malignancies commonly express estrogen receptor (ER) and/or progesterone receptor (PR) and endocrine therapy is often considered as treatment for advanced, potentially hormone-sensitive gynecologic cancers, especially low-grade endometrial and ovarian tumors. Endocrine therapy blocks ER signaling through a variety of strategies, most commonly estrogen-deprivation, as with aromatase inhibitors (AI), or direct-antagonism of ER through selective ER-modulators (SERMs) or -degraders (SERDs).

Failure of endocrine therapy occurs through intrinsic or acquired resistance mechanisms. Mutations in ERα (encoded by *ESR1*) are a mechanism of resistance to endocrine therapy commonly observed in metastatic breast cancer. These mutations occur predominantly in the ligand-binding domain (LBD) of the receptor and result in constitutive activation of ERα in the absence of estrogen. Activating *ESR1* mutations (*mutESR1*) most frequently occur in a hotspot region encompassing amino acids 536–538 within the ERα LBD [1,2], with a smaller number occurring at other LBD sites, namely E380, V422, S463, and L469 [3–5]. In breast cancer, *mutESR1* arise in 25–50% of patients who receive endocrine therapy, especially AIs, but are relatively rare (3%) in primary tumors [1,4,6–8]. Thus, *mutESR1* are primarily an acquired resistance pathway to endocrine therapy that may also account for rare cases of intrinsic resistance.

ESR1 amplification has also been suggested as a mechanism of resistance to endocrine therapy resulting in worse outcomes [9,10]. *ESR1* amplification is reported in early pre-cursor lesions of endometrial cancer [11–13]. However, the clinical impact of *ESR1* amplifications is controversial and detection methods may result in overcalling of this genomic alteration [14].

A study evaluating ERα as a predictive biomarker in endometrioid endometrial cancer identified 19 cases of *mutESR1* in 1034 samples (1.8%) [15]. This study focused solely on mutations arising in codons 536–538, was limited to endometrioid endometrial cancers, and did not provide clinical information regarding prior endocrine therapy or response to therapy.

The purpose of the current study was to quantify the frequency of *ESR1* genomic alterations, including *mutESR1*, identified by comprehensive genomic profiling in gynecologic malignancies. We present our clinical experience treating tumors with both de novo and acquired *mutESR1*. The effect of selected SERMs and SERDs on the transcriptional activity of individual *mutESR1* was examined.

2. Results

2.1. ESR1 genomic profiles in gynecologic malignancies

Of the 9645 clinical samples from gynecologic malignancies evaluated with CGP in this study, 285 (3%) samples contained a total of 295 *ESR1* alterations, including substitutions, amplification, and rearrangements. Ten cases exhibited two separate alterations each. The types and frequency of alterations by site of disease origin are listed in Table 1. Substitutions were the most common *ESR1* alteration identified (194/295, 66%), with 44% (86/194) of those occurring in *ESR1* codons expected to result in ERα constitutive activity. Of the activating variants, alterations in codons 536–538 were most common accounting for 25% (75/295) of all *ESR1* alterations and occurring in 0.8% (75/9645) of cases. Another 12 cases (0.1%) contained V422del, S463P, or L469 mutations. *ESR1* amplifications were the next most common genomic alteration identified (80/295, 27%) and were present in 0.8% of cases. Median *ESR1* copy number in amplified cases was 8 (range 6–38). *ESR1* rearrangements were present in 0.2% of cases (Table S1).

Types and frequency of *mutESR1* by histologic subtype are listed in Table 2. *mutESR1* were more common in uterine cancers (63/3101, 2%) compared to other primary sites (24/6530, <1%, *p* < 0.0001). *mutESR1* were enriched in carcinomas with endometrioid histology:

Table 1
Types and frequency (%) of ESR1 alterations identified in gynecologic malignancies by primary site.

Type of alteration	Frequency N = 9645[a]	Ovary/FT N = 5594	Uterus N = 3101	Cervix N = 720	Vulva/ vagina N = 216
Total	295 (3.1)[b]	120 (2.1)	160 (5.2)	9 (1.2)	6 (2.8)
Amplification	80 (0.8)	45 (0.8)	34 (1.1)	1 (0.1)	–
Deletion	1 (<0.1)	–	1 (<0.1)	–	–
Fusion	2 (<0.1)	1 (<0.1)	–	–	1 (0.5)
Rearrangements	18 (0.2)	9 (0.2)	9 (0.3)	–	–
Total substitution variants	194 (2.0)	65 (1.2)	116 (3.7)	8 (1.1)	5 (2.3)
Codon 536–538	75 (0.8)	18[c] (0.3)	56[c] (1.8)	1 (0.1)	–
Other activating Mut	12 (0.1)	3 (0.0)	7 (0.2)	–	2 (0.9)

"–": none present. Abbreviations: FT: fallopian tube, Mut: mutation.
[a] No *ESR1* alterations were identified among 14 cases of placental cancers included in total number of cases assessed.
[b] Includes 10 cases with 2 alterations each.
[c] 1 ovarian case and 2 uterine cases had 2 codon 536–538 mutations each.

4.4% (24/548) in uterine endometrioid vs 0.2% (1/446) in uterine serous carcinomas (*p* < 0.0001) and 3.5% (5/144) in ovarian endometrioid compared to 0.3% (12/3502) in ovarian serous carcinomas (*p* = 0.0004). Two uterine endometrioid carcinomas exhibited the following co-occurring *ESR1* mutations: Y537N with Y537S, and L536H with Y537C, respectively. An ovarian serous carcinoma exhibited both *ESR1* Y537S and D538G. Grade was not available for any of the uterine cases and absent for 89% of the ovarian cases, thus an assessment based on grade could not be performed. Uterine endometrial stromal sarcomas (ESS) had a proportionally higher frequency of *mutESR1* than uterine leiomyosarcomas (LMS) [3/103 (3%) vs. 3/421 (0.7%), respectively], though this did not reach statistical significance (*p* = 0.09).

To further examine the prevalence of *mutESR1* in human ovarian and endometrial cancers, publically available databases were explored [16–21] using the cBioPortal [22,23] and COSMIC [24]. In total 41 gynecologic malignancies with *mutESR1* were identified (Table S2). The majority (37/41, 90%) occurred in uterine tumors including 2 cases of endometrial stromal sarcoma. No cases were identified in the cervical cancer TCGA database or the ovarian cancer TCGA database, which comprises exclusively high-grade serous cancer cases. One endometrial case had 2 *mutESR1* alleles (V422del and Y537H). Interestingly, the majority of the *mutESR1* occurred in endometrioid tumors (34/41, 83%). An additional uterine endometrioid case was identified with an *ESR1* P535H

Table 2
mutESR1 identified in gynecologic malignancies by histologic subtype.

Primary site & histology	N	*mutESR1* N (%)	Y537S/D538G	Other activating variants
Cervix	720	1 (0.1)		
Clear cell	15	1 (6.7)	–	1
Ovary/FT	5594	21 (0.4)		
Carcinoma NOS	1079	4 (0.4)	2	2
Endometrioid	144	5 (3.5)	2	3
Serous	3502	12 (0.3)	12	–
Uterus	3101	63 (2.0)		
Carcinoma NOS	1063	27 (2.5)	10	17
Endometrioid	548	24 (4.4)	10	14
Clear cell	78	1 (1.3)	–	1
Papillary serous	446	1 (0.2)	–	1
Carcinosarcoma	303	4 (1.3)	4	–
Leiomyosarcoma	421	3 (0.7)	2	1
ESS	103	3 (3.0)	3	–
Vulva/vagina	216	2 (0.9)		
SCC	134	1 (0.7)	–	1
Adenocarcinoma	32	1 (3.1)	–	1
Total	9645[a]	86 (0.9)	45 (52.3)	41 (47.7)

"–": none detected. Abbreviations: FT: fallopian tube, ESS: endometrial stromal sarcoma, SCC: squamous cell carcinoma, NOS: not otherwise specified.
[a] No *ESR1* alterations were identified among 14 cases of placental cancers included in total number of cases assessed.

mutation. This mutation has not been functionally characterized and it was not included in the analyses.

The overall frequency of cases with *mutESR1* in the publically available databases was similar to that seen in the CGP dataset. In the AACR Project GENIE databases, *mutESR1* were present in 2.3% (32/1363) of uterine endometrial cancers, 0.1% (2/1733) of ovarian cancers, 0.4% (1/279) of cervical cancers, and 0.9% (2/234) of uterine sarcomas. Similarly, *mutESR1* were enriched in endometrioid endometrial cancers [30/654 (4.6%) endometrioid vs 0/244 serous, $p = 0.0001$] and endometrioid ovarian cancers [2/70 (2.9%) endometrioid vs. 0/838 high-grade serous, $p = 0.006$]. *mutESR1* was enriched in ESS compared to LMS (2/16 vs 0/129, respectively, $p = 0.012$) in the AACR Project GENIE uterine sarcoma dataset. Of the 248 cases with mutation data in the TCGA uterine corpus dataset, 5 (2.0%) contained *mutESR1*, all with endometrioid histology; however the comparison between endometrioid and serous histology did not show a statistically significant difference [5/200 (2.5%) endometrioid vs 0/44 serous, $p = 0.59$].

In sum, combining our CGP dataset and the publically available datasets, 125 gynecologic malignancies with 129 individual *mutESR1* were identified. Variants most commonly occurred in the known hotspot region, with 29/129 (22.5%) in codon 536, 59/129 (45.7%) in codon 537, and 24/129 (18.6%) in codon 538. Fig. 1 presents a schematic overview and frequency distribution of the *mutESR1* identified. Y537S (42/129, 32.6%) and D538G (24/129, 18.6%) were the most common individual *mutESR1* identified.

2.2. Clinical relevance of mutESR1 in gynecologic malignancies and response to treatment

Detailed clinical information was available for 8 patients with *mutESR1*. Fig. 2 and Table 3 illustrate the clinical course for patients identified with *mutESR1* tumors. In 5 of 8 cases, *mutESR1* was identified after AI exposure. Serial sampling performed in 3 showed the emergence of *mutESR1* (Patients A and G) or an increase in allelic frequency (Patient D). Patient D had two exposures to AI: one of short duration (1 month) and a subsequent exposure lasting 7 months. Because no pre-AI sample is available, when the *mutESR1* first evolved cannot be determined. However, the allelic frequency of *ESR1* Y537S was increased after the second exposure (37% post-7-months vs 4% post-1-month, 9.25-fold increase) compared to *KRAS* (40% post-7 months vs 23% post-1 month, 1.74-fold increase).

Table S3 outlines the clinical benefit duration (CBD) achieved with SERM/SERD therapy compared to the immediate antecedent therapy. Of the 8 cases, 6 experienced a greater duration of benefit with ER-directed therapy than chemotherapy. In some cases, ER-directed therapy led to extended benefit. In particular, a 58-year-old woman (Patient C) with Stage IIIC primary peritoneal low-grade serous carcinoma had no tumor response to neoadjuvant chemotherapy. She underwent cytoreductive surgery and CGP revealed an *ESR1* Y537N mutation. The patient had no prior history of endocrine therapy. Immunohistochemistry (IHC) showed this mutation was associated with high ERα expression (Fig. 2B). Given the concern for intrinsic resistance to AI therapy conferred by the mutation, treatment with fulvestrant was started and within 4 months she experienced a major CA125 biochemical response (Fig. 2B). She has had prolonged clinical benefit of >4 years with minimal residual disease based on radiologic imaging. In other cases, switching therapy to an alternate SERM or SERD after progression on the initial ER-targeted therapy also provided clinical benefit (Patients A and B). Patient A experienced a combined total of 20 months CBD with tamoxifen followed by fulvestrant compared to 2.5 months CBD with the antecedent chemotherapy. Therefore, despite the presence of an activating *ESR1* mutation, therapy with ER-targeting agents can be beneficial for some patients with *mutESR1* associated gynecologic malignancies.
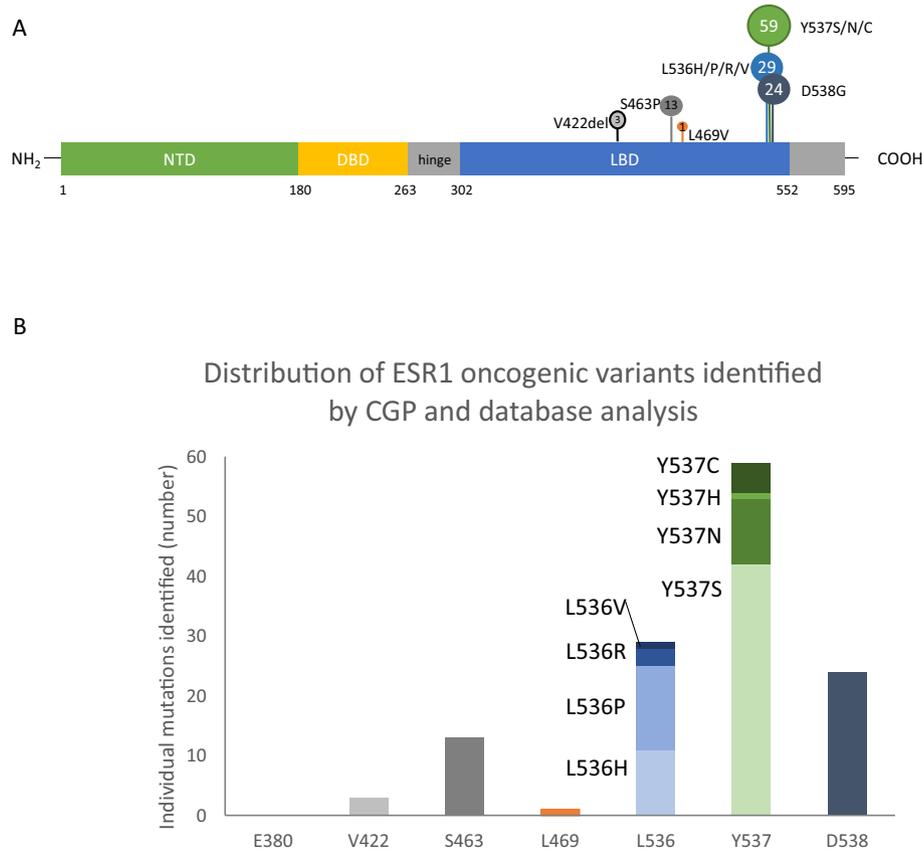

Fig. 1. Schematic overview of mutESR1 identified in gynecologic malignancies. (A) Distribution of mutations identified. (B) Frequency of individual variants identified. N = 125, DBD: DNA binding domain, LBD: ligand binding domain.
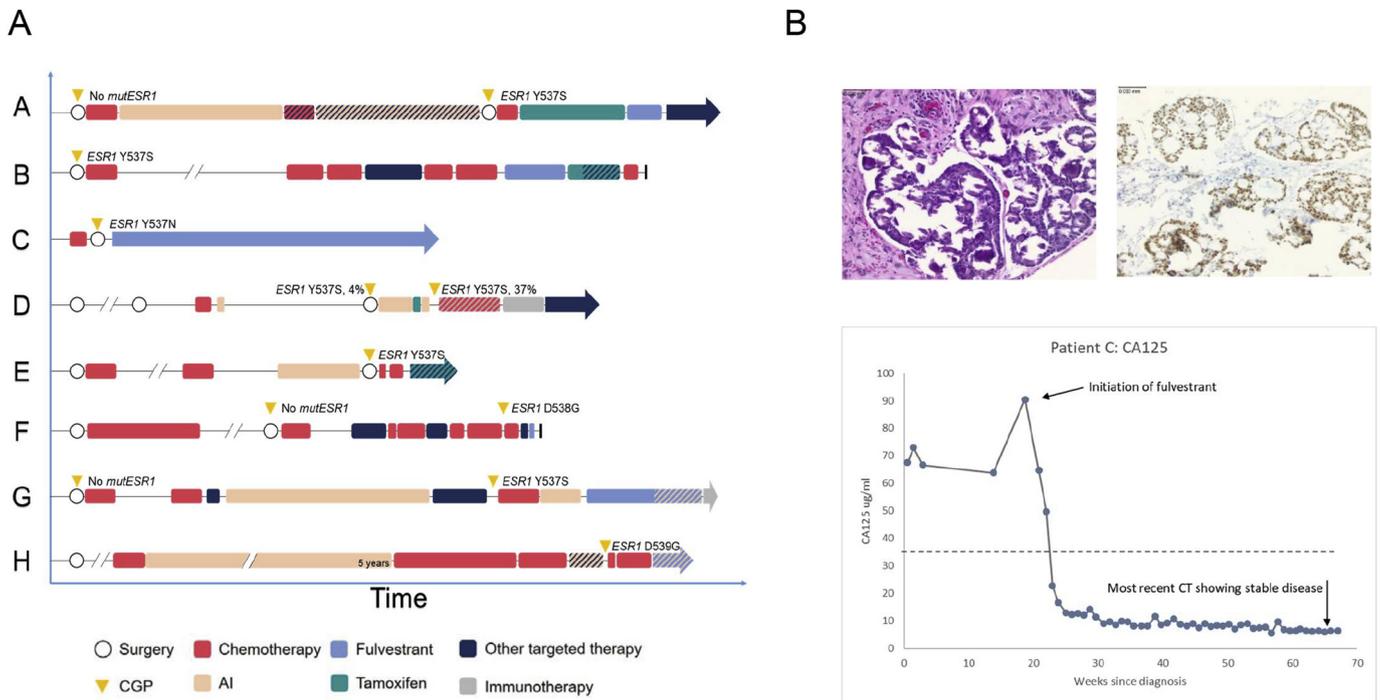
Fig. 2. Clinical relevance of *mutESR1* in gynecologic malignancy. A. Clinical course of patients identified with gynecologic malignancies harboring *mutESR1*. Eight individual patients (A–H) with *mutESR1* were identified. Each box or wide arrow delineates a treatment received colored according to the legend and the width reflects relative duration of therapy. A wide arrow represents ongoing therapy. Hashed boxes/arrows reflect combined therapy. The triangle reflects when the sample evaluated by CGP was procured. Percentages reflect allelic frequency of the mutation within the sample. B. (top) Expression of ERα in tumor harboring *ESR1* Y537N. H&E stain (left) and ERα by immunohistochemistry (right). (bottom) CA125 response after initiation of fulvestrant (arrow). Upper limit of normal of the CA125 test (dashed horizontal line).

2.3. mutESR1 confers partial resistance to endocrine therapies in ovarian cancer cells

In breast cancer cell lines, *mutESR1* exhibit decreased sensitivity to tamoxifen and fulvestrant. We evaluated the transcriptional activity in response to SERMs or SERDs of the most common *mutESR1* identified in our series using a reconstituted ERE-luciferase reporter assay in CAOV2 ovarian carcinoma cells. ERα constructs were generated containing 3 different amino acid substitutions at position 537 (Y537C, Y537N, Y537S) and 1 at position 538 (D538G). The wild-type ERα receptor (WT) was only activated in the presence of 17-beta estradiol (E2). Each of the mutants exhibited substantial constitutive activity in the absence of E2 when compared to the activity of ERα WT (Fig. 3A). The constitutive activity of each of the receptors was similar to the maximally E2-stimulated activity of the WT receptor, with the exception

that *ESR1* Y537S showed statistically significant increased activity (134% compared to WT receptor, $p = 0.0137$).

The ability of clinically available SERMs [tamoxifen, tested in this study as its active metabolite, 4-hydroxytamoxifen (4OHT); raloxifene; bazedoxifene; and lasofoxifene] and SERDs [fulvestrant (ICI) and GW7604] to inhibit ERα mutant transcriptional activity was evaluated in ovarian cancer cells. In the CAOV2 ovarian cancer cell model, each of the drugs was able to effectively inhibit mutant transcriptional activity, albeit with reduced potency when compared to the WT receptor. Fig. 3B and C are tabular and graphical presentations of the inhibitory concentrations required to reduce transcriptional activity by 50% and 90% (IC50 and IC90, respectively). Differences in potency between the WT receptor and individual mutations varied considerably by drug (Table S4). For example, the 4OHT IC50 required for Y537C is 9× higher than for WT, while the ICI IC50 required for Y537C was >1600× that required for WT.

Table 3
Clinical characteristics of patients identified with mutESR1 gynecologic malignancies.

Patient	Age	Disease site (histology)	CGP sample (*ESR1* mutation, % allelic frequency[a])	Other mutations (% allelic frequency[a])	Prior aromatase inhibitor (timing & duration)	Benefit of SERM/SERD therapy
A	24	Ovary (LG serous)	PCS (none)	None	No	Yes
			SCS (Y537S)	None	Yes (2 courses, 24 mths each)	
B	35	Synchronous endometrial and ovary (LG endometrioid)	PCS (Y537S)	BTK, CEBPA, CTNNB1, IGF1R, MED12, NOTCH3	No	Yes
C	58	Primary peritoneal (LG serous)	ICS (Y537N)	None	No	Yes
D	43	Ovary (LG serous)	SCS (Y537S, 4%)	KRAS (23%)	Yes (1 month prior to SCS)	No
			Biopsy after 2nd AI (Y537S, 37%)	KRAS (40%)	Yes (7 mths after SCS)	
E	59	Ovary (HG endometrioid)	SCS (Y537S)	APC	Yes (12 mths)	Yes
F	40	Ovary (HG serous)	SCS (none)	BRCA1, TP53	No	No
			Biopsy after multiple chemo (D538G)	BRCA1, TP53		
G	59	Ovary (LG → HG serous)	PCS (none)	TP53, CIC	Yes (30 mths)	Yes
			Biopsy of HG serous recurrence (Y537S)	TP53, CIC		
H	59	Endometrial (LG endometrioid)	Biopsy following AI (D539G)	AKT1, CTNNB1, ARID1A, MLL	Yes (60 mths)	Yes

[a] Percent allelic frequency reported if known. LG: low-grade, HG: high-grade, PCS: primary cytoreductive surgery, SCS: second cytoreductive surgery, ICS: interval cytoreductive surgery (after neoadjuvant chemotherapy), chemo: chemotherapy.
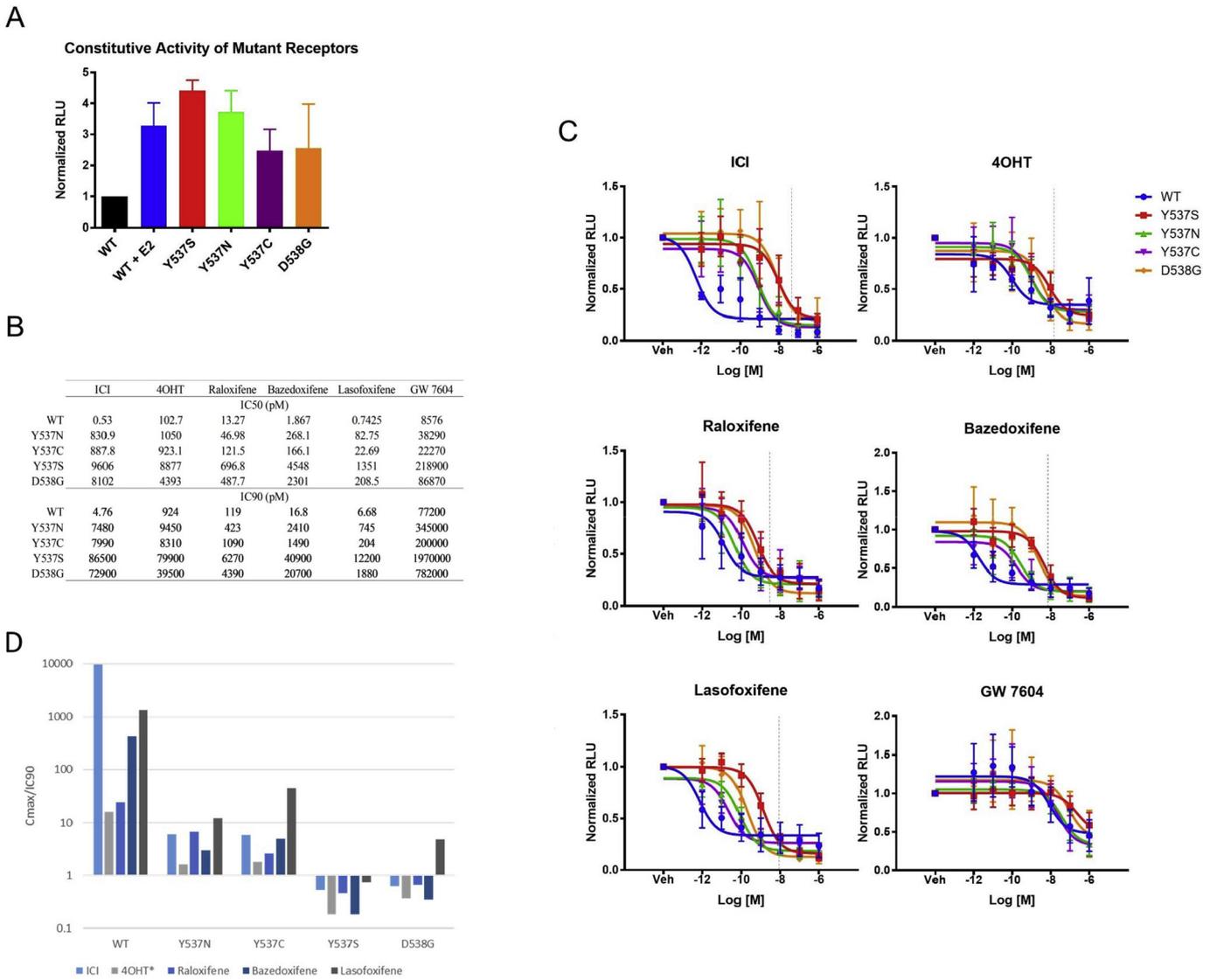
A

Constitutive Activity of Mutant Receptors

B

	ICI	4OHT	Raloxifene	Bazedoxifene	Lasofoxifene	GW 7604
			IC50 (pM)			
WT	0.53	102.7	13.27	1.867	0.7425	8576
Y537N	830.9	1050	46.98	268.1	82.75	38290
Y537C	887.8	923.1	121.5	166.1	22.69	22270
Y537S	9606	8877	696.8	4548	1351	218900
D538G	8102	4393	487.7	2301	208.5	86870
			IC90 (pM)			
WT	4.76	924	119	16.8	6.68	77200
Y537N	7480	9450	423	2410	745	345000
Y537C	7990	8310	1090	1490	204	200000
Y537S	86500	79900	6270	40900	12200	1970000
D538G	72900	39500	4390	20700	1880	782000

C

D

Fig. 3. ERα LBD mutations confer constitutive transcriptional activity and alter receptor sensitivity to SERMs/SERDs. A. Luciferase assay measuring transcriptional activity of the WT and mutant receptors. WT + E2 is in the presence of 10^{-8} M of 17β-estradiol. B. Tabular presentation of IC50 and IC90 of each receptor:drug combination in picomolar (pM) C. Inhibition dose-response curves for each anti-estrogen. All inhibition curves were done in the presence of 10^{-9} M (1 nM) 17β-estradiol. Data normalization is performed with respect to the vehicle treatment for each individual receptor. These plots include data from five independent experiments and each value is an average of triplicates from each experiment. Dashed vertical line in the ICI, raloxifene, bazedoxifene, and lasofoxifene graphs represents the maximal clinically achievable concentration (C_{max}) for each drug respectively [25–28]. For 4OHT, the median serum concentration is presented for 4OHT [29]. D. Comparison C_{max}/IC90 ratio across *mutESR1*. The maximum achievable blood concentration (C_{max}) for each ER antagonist was identified in the literature [25–28] and divided by each receptor's IC90 as determined by the dose-response curves in the luciferase assays. *The median serum concentration was used for 4OHT [29]. The values are reported on a logarithmic scale.

Interestingly, the mutant receptors themselves exhibited differential responses to the drugs (Fig. S1). The IC50 for each of the drugs on the ERα Y537N and Y537C mutants were typically similar and within ~2 fold of each other (Table S5). In contrast, the IC50 for the ERα Y537S and D538G mutants were more likely to be similar and frequently higher than the IC50 for either of the other two mutants.

The IC50 and IC90 of each receptor:antagonist pair was compared in our assays to the antagonist's reported maximum achievable blood concentration in humans (C_{max}) (Table S5) [25–28]. Because the C_{max} of the tamoxifen metabolite, 4OHT, was not available, the median serum concentration of 4OHT measured in patients receiving tamoxifen 20 mg daily was used [29]; the typical dose of tamoxifen used for the treatment of gynecologic malignancies is 20 mg twice a day. Fig. 3D is a visual representation of the C_{max}:IC90 and shows that the concentration to reach the IC90 is achievable with each of the antagonists for the Y537N and Y537C mutations, while only lasofoxifene would be

expected to achieve the IC90 for the D538G mutation and none reach the IC90 for Y537S.

3. Discussion

This study demonstrated that activating mutations within the *ESR1* LBD occur in gynecologic malignancies. This finding has important treatment implications. ERα-dependent malignancies harboring these mutations are unlikely to respond to estrogen deprivation therapies, such as AIs, because these alterations confer constitutive transcriptional activity to ERα. The frequency of *mutESR1* in the current study is low, only 0.9% in unselected cases. However, these mutations are enriched in endocrine-responsive subtypes of gynecologic malignancies: specifically, the endometrioid histologic subtype of endometrial and ovarian cancers. Identification of *mutESR1* in up to 5% of endometrioid endometrial cancers is higher than previously reported (1.8%) [15]. The

difference may be due to sampling of more recurrent cases or post-treatment cases in the current dataset. Another endocrine-sensitive histologic subtype, ESS, also exhibited a higher frequency of *mutESR1* than its counterpart, LMS, however this was statistically significant in only one dataset. Four cases exhibited two *mutESR1* consistent with reports of heterogeneity and polyclonality of *mutESR1* in breast cancer [30]. The frequency of *ESR1* amplifications in the current dataset, specifically in the endometrial cancer cohort, is much lower than previously reported [11–13]. The clinical relevance of *ESR1* amplifications is still unknown.

Endocrine therapy is preferentially used in low-grade gynecologic malignancies. Whether *mutESR1* are enriched in low-grade gynecologic malignancies could not be determined because of the limited information regarding tumor grade in the current study. However, *mutESR1* appear to be enriched in histologic subtypes likely to have a higher proportion of low-grade cases (i.e. endometrioid vs serous histology), supporting the hypothesis. Further studies comparing the prevalence of *mutESR1* in high-grade and low-grade subtypes are needed. Nevertheless, these variants are more likely to be clinically relevant in the subset of tumors for which endocrine therapy is commonly utilized.

In breast cancer, *mutESR1* have been demonstrated to arise primarily as a resistance mechanism to estrogen deprivation therapy resulting in ligand-independent ERα signaling. The current study is limited by the lack of information regarding prior treatment history from the majority of patients whose tumors underwent CGP. Thus it is impossible to determine whether *mutESR1* emerge in gynecologic malignancies as a result of exposure to endocrine therapy. However, at least three of the clinical cases support the hypothesis that these mutations are selected for or emerge as resistance mechanisms to AI therapy. This is consistent with a recent case report of a patient with low-grade serous cancer who was found to have a Y537S mutation in a metastatic lesion that progressed after treatment with AI [31]. Interestingly, *mutESR1* in breast cancer appear more likely to develop after AI therapy in the metastatic versus adjuvant treatment setting [32,33]. In a recent study of adjuvant AI for low-grade serous ovarian cancer, no *mutESR1* were identified in the small proportion of patients who developed recurrent disease [34]. Thus, *mutESR1* may be more likely to develop in patients treated with AIs for metastatic disease.

Conversely, three cases (Patients B, C and F) had no known history of endocrine therapy prior to identification of *mutESR1*. For Patient B, the mutation was present in tumor at diagnosis, suggesting that the mutation arose de novo and possibly played a role in the pathogenesis of the tumor. Similarly, the *mutESR1* identified in the TCGA cases were from samples collected at the time of diagnosis. However, whether patients may have had prior exposure to anti-estrogens for treatment of a separate malignancy, such as breast cancer is not known. Nonetheless, these mutations may develop independent of AI exposure and result in intrinsic resistance to estrogen-deprivation therapies.

Because of the constitutive activity conferred by the mutation, tumors with *mutESR1* are unlikely to respond to AI therapy. The in vitro studies support prior reports that higher doses of anti-estrogens are required to inhibit mutant ERα [1,8]. However, *mutESR1* breast cancers can respond to tamoxifen and fulvestrant supporting that efficacious doses can be achieved clinically [2,4]. For Patient C, early identification of the mutation has led to long-term clinical benefit with fulvestrant, extending past 52 months at the time of submission of this manuscript. Others also received clinical benefit, though this was not universal. Overall, SERM/SERD therapy has the potential to provide benefit despite the presence of a *mutESR1*.

What accounts for the variable differences in benefit among the clinical cases of *mutESR1* is unknown. Differences may be due to 1. the use of hormone therapy in a later phase of the disease course after development of multiple adaptive/resistance mechanisms; 2. the influence of co-occurring mutations; or 3. the specific *mutESR1* present within each tumor (e.g. Y537N vs Y537S). Supporting the third hypothesis, the transcriptional data demonstrated that the efficacy of inhibition of

ERα transcriptional activity with various SERMs/SERDS differs by individual mutation. This is similar to the findings of other groups [5,35] who showed differences in response to SERMs/SERDs between the Y537S and D538G mutations. The current data shows that different amino acid substitutions at the same site (Y537C vs Y537N vs Y537S) also exhibit different IC50 to individual drugs. Furthermore, exposure (C_{max}) of some agents exceeds the IC90 of some, but not all, mutated receptors. It is important to recognize the limitations of this data: nuances in transcriptional response in vitro may not translate to clinical efficacy given the complexities of tumor proliferative signals and individual patient pharmacokinetic and pharmacodynamic considerations.

The finding of *mutESR1* in gynecologic malignancies has several important clinical implications. First, *mutESR1* may arise de novo in gynecologic malignancies in the absence of prior exposure to endocrine therapy. Endocrine therapy, especially AIs, may increase the prevalence of these mutations. Thus, traditionally endocrine-responsive gynecologic malignancies should be assessed for these mutations. This may be especially important for tumors that develop resistance or are refractory to endocrine therapy. Second, the functional and clinical data supports the use of alternative endocrine therapy, namely SERMs or SERDs, in some patients with *mutESR1* gynecologic malignancies. Thus, the identification of a *mutESR1* does not preclude further treatment with selected endocrine therapy, though relative response may be affected by the individual mutation present. Given the low frequency of these mutations in gynecologic malignancies, a large scale effort will be required to delineate the prevalence of *mutESR1* across gynecologic malignancies and conditions under which they arise, with an emphasis on malignancies considered for treatment with endocrine therapies. The recent development of technology that can be used to evaluate mutation status in plasma circulating tumor DNA may be useful to non-invasively monitor tumor mutation status over time and in response to treatment [36]. This approach has identified greater heterogeneity and polyclonality in the development of *mutESR1* than had been appreciated with tissue evaluation from individual mutation sites [2,30,36]. Finally, prospective studies using *ESR1* mutation status to direct endocrine therapy should be undertaken to understand how these mutations may be used to guide therapeutic decision making and personalize care for patients with gynecologic malignancies.

4. Materials and methods

4.1. Comprehensive genomic profiling

Comprehensive next generation sequencing-based genomic profiling (CGP) was performed for 9645 cases of gynecologic malignancies involving the ovary, fallopian tube, uterus, cervix, placenta, vulva, or vagina during the course of routine clinical care. Approval for this study, including a waiver of informed consent and HIPAA waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817). The pathologic diagnosis of each case was confirmed on routine hematoxylin and eosin (H&E) stained slides and all samples forwarded for DNA extraction contained a minimum of 20% tumor nuclear area. From each sample, ≥50 ng DNA was extracted from 40 μm of tumor samples provided as formalin-fixed, paraffin-embedded (FFPE) tissue blocks.

The sequencing methods used for CGP have been described in detail elsewhere [37,38]. Sample processing and sequencing analysis was performed in a Clinical Laboratory Improvement Amendments (CLIA)- and College of American Pathologists (CAP)-accredited laboratory (Foundation Medicine). Samples were assayed using adaptor-ligation and hybrid capture (Agilent SureSelect custom kit) next-generation sequencing (FoundationOne®); genes assayed by version are listed in Tables S6–9. Sequencing of captured libraries was performed using Illumina HiSeq technology to a mean exon coverage depth of >500×, and resultant sequences were analyzed as previously described

[37,38]. The bioinformatics processes used in this study have been previously reported [37,38].

4.2. Identification of ESR1 mutations in public databases

The cBioPortal [22,23] was used to access *ESR1* mutation data from the cervical TCGA [21], endometrial TCGA [20], ovarian TCGA [19], uterine carcinosarcoma [17], and AACR Project GENIE datasets [18]. COSMIC (cancer.sanger.ac.uk) [24] was used to identify an additional sample with an *ESR1* mutation from an ovarian cohort [16].

4.3. Clinical evaluation of gynecologic malignancies with mutESR1

Three cases were identified during review of medical records under an Institutional Review Board protocol (with waiver of informed consent and a HIPAA waver of authorization) at the Duke University Medical Center aimed at evaluating the utility of evaluation of ER in the course of routine clinical care of patients with low-grade ovarian or endometrial cancers treated at Duke University between January 1, 2000 and June 30, 2016. The other three cases were identified by searching the Clearity Foundation Ovarian Cancer Data Repository, which contains clinical histories and tumor molecular profiling data collected with informed patient consent under a Western Institutional Review Board-approved protocol. Two cases from Mayo clinic were deemed exempt by the IRB. Demographic, tumor characteristics, treatment and response data were extracted for all cases.

4.4. Site-directed mutagenesis

Exsite mutagenesis was performed using the corresponding primers in Table S10 on a pENTR2B ERα WT construct using Pfu-ultra Taq polymerase and primers were PNK phosphorylated. Following PCR amplification, products were digested with *Dpn*I at 37 °C for 1 h, followed by overnight ligation at 16 °C. Ligated products were transformed into DH5α bacterial cells and grown on kanamycin-resistant plates. The pENTR clones were verified by sequencing and then swapped into the pcDNA-DEST vector using the Gateway system (Invitrogen) for expression analysis.

4.5. Reporter gene assay

The following anti-estrogens were obtained through commercially available sources: 17β-estradiol and 4-hydroxytamoxifen (Sigma-Aldrich), ICI 182,780 and raloxifene (Tocris), bazedoxifene and lasofoxifene (MedChem Express). GW7604 was synthesized by the Duke Small Molecule Synthesis Facility. CAOV2 cells were co-transfected with the 7X-ERE-TK luciferase reporter gene [39] and expression constructs for either wild-type or mutant receptors using Fugene transfection reagent (Promega). pCMV-B-gal was used as a control for transfection efficiency and pcDNA was added for a final DNA concentration of 75 ng per triplicate group. Cells were treated with indicated ligand 5 h post-transfection. Following 24 h of treatment, cells were lysed and the luciferase and β-gal assays were performed as described previously [40]. Quantification was performed using the Fusion α -FP HT plate reader (PerkinElmer Life Sciences).

4.6. Statistics

Data was analyzed using GraphPad Prism. *p* values for the comparison of *mutESR1* frequency by primary site and histologic subtypes was performed using Fisher's exact test. Dose-response curves for the transcriptional assays were determined by log(inhibitor) vs. response (three parameters). *p* values for comparison of the constitutive activity of the mutations were performed using an unpaired *t*-test.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.04.010.

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Manuscript writing: All authors.

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Conflict of interest statement

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