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Clinically Observed Estrogen Receptor Alpha Mutations within the Ligand-Binding Domain Confer Distinguishable Phenotypes

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Abstract

Objective—20–50% of estrogen receptor-positive (ER+) metastatic breast cancers express mutations within the ER ligand-binding domain (LBD). While most studies focused on the constitutive ER signaling activity commonly engendered by these mutations selected during estrogen deprivation therapy, our study was aimed at investigating distinctive phenotypes conferred by different mutations within this class.

Methods—We examined the two most prevalent mutations, D538G and Y537S, employing corroborative genome-edited and lentiviral-transduced ER+ T47D cell models. We used a luciferase-based reporter and endogenous phospho-ER immunoblot analysis to characterize the estrogen response of ER mutants and determined their resistance to known ER antagonists.

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Disclosure Statement

The authors declare that they have no competing interests.

Use of Animal and Human Subjects

The authors declare that these studies did not involve the use of either human subjects or animal model experiments.

Results—Consistent with their selection during estrogen deprivation therapy, these mutants conferred constitutive ER activity. While Y537S mutants showed no estrogen dependence, D538G mutants demonstrated an enhanced estrogen-dependent response. Both mutations conferred resistance to ER antagonists that was overcome at higher doses acting specifically through their ER target.

Conclusions—These observations provide a tenable hypothesis for how D538G ESR1-expressing clones can contribute to shorter progression-free survival observed in the exemestane arm of the BOLERO-2 study. Thus, in those patients with dominant D538G-expressing clones, longitudinal analysis for this mutation in circulating free DNA may prove beneficial for informing more optimal therapeutic regimens.

Keywords

Estrogen receptor; Estrogen dependence; Metastatic breast cancer; Ligand-binding domain mutations

Introduction

Cancer is the number one killer of Americans, with the World Health Organization indicating that cancer cases are expected to surge 57% worldwide in the next 20 years [1]. A major reason for this continued mortality is the ability of the cancer genome to undergo dynamic evolution with resulting somatic mutations conferring phenotypes on particular tumor clones that enable adaptation to an otherwise non-permissive environment. Natural selection of these tumor sub-clone populations gives rise to landmarks of disease progression such as resistance to drug therapy and the acquisition of invasive or metastatic potential. Metastatic breast cancer is a striking example of such an evolving disease where despite significant progress in the treatment of the primary breast cancer, metastatic breast cancer remains a lethal disease, responsible for more than 40,000 deaths per year in the US alone [2]. Insidiously, breast cancers recur late, often more than 5 years after initial treatment, allowing a prolonged period for evolution and clonal selection [3, 4].

During the course of our studies on the evolution of metastatic breast cancer, we along with other groups identified several somatic base-pair missense mutations in estrogen receptor alpha (ESR1; ER) expressed in the metastases of patients with advanced ER+ breast cancer who had undergone mainstay estrogen deprivation therapy with aromatase inhibitors [5–15]. These mutations clustered within the ligand-binding domain (LBD) of the ER. To enable the clinical significance of these ESR1 mutations to be determined prospectively, we utilized a highly sensitive digital droplet-based PCR method to quantify these mutations in primary tumors, metastatic lesions, and importantly in the circulating cell-free DNA (cfDNA) of liquid biopsies [5]. Consistent with other studies, these ESR1 mutations were detected at very low prevalence and allele frequencies in primary tumors and much higher prevalence and allele frequencies in metastases, suggesting that in some tumors rare ESR1 mutant clones may be enriched by estrogen deprivation therapy [16–20]. Indeed, in complementary studies expression of these mutations conferred constitutive ER activation implying that in the relative absence of estrogen, ER signaling *per se* represents an important metastatic dependency. In some patients monoclonal ESR1 mutations were present while in other

patients we observed polyclonal ESR1 mutations [5]. Interestingly, longitudinal analysis of cfDNA in a patient with three different ESR1 mutations demonstrated increased mutant allele frequencies for two clones and loss of the third clone, possibly reflecting differential response of individual ESR1 mutations to treatment [5]. These observations suggested the hypothesis that distinct ESR1 mutations within the LBD of ER might confer distinguishable and clinically relevant phenotypes.

Understanding possible differences among the phenotypes conferred by these ESR1 LBD mutations would have the potential to increase 1) the impact of monitoring ESR1 mutations as a prognostic indicator of disease progression and predictive biomarker of resistance to treatment 2) our knowledge of ER signaling dependencies in metastatic breast cancer, 3) our ability to design next generation ER antagonists, and 4) the adaptive optimization of therapeutic strategies in individual patients. Therefore, we have undertaken a quantitative analysis to determine phenotypic differences conferred by individual ESR1 mutations. We have examined multiple clones of the two most common ESR1 mutations observed in the clinic (D538G and Y537S) in a parental human breast cancer cell line (T47D) utilizing both lentiviral transfection and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing [21]. We used an estrogen response element (ERE) transactivation luciferase-based reporter assay and a corroborative endogenous phospho-ER immunoblot analysis to determine the estrogen response of each ESR1 mutant expressing cell line and their respective sensitivity to approved and investigational selective estrogen receptor degraders (SERDs) and modulators (SERMs). These studies describe key phenotypic differences among clinically observed ESR1 mutations and form the basis for our continuing efforts to understand the mechanistic underpinnings of ER+ metastatic disease and use this knowledge to identify more effective therapies.

Materials and Methods

Reagents

Fulvestrant, 4-Hydroxytamoxifen (4-OHT), and β -Estradiol (E2) were obtained from Sigma Aldrich. Peruvoside was obtained from Santa Cruz Biotechnology Inc. and AZD 9496 was obtained from Fisher Scientific.

Plasmids

3X ERE-TATA luciferase (developed by Donald McDonnell (Addgene plasmid # 11354); [22] and pRL-SV40P luciferase (developed by Ron Prywes (Addgene plasmid # 27163); [23] plasmids were purchased from Addgene. For the preparation of plasmids for transfection, a 2 mL starter culture was grown for 8 hours at 37°C in LB (Lennox) + 100 μ g/mL of ampicillin (Fisher Scientific). 0.5 mL of the starter culture was then used to inoculate 250 mL of LB-ampicillin for overnight growth. Purified plasmid was isolated using an endo-free Maxi-kit (Qiagen) according to manufacturer's protocol. Plasmid DNA sequence was confirmed by direct sequencing and by restriction endonuclease digestion with XhoI and PacI (New England Biolabs). Plasmid concentration was then determined using a NanoDrop Lite spectrophotometer (ThermoFisher).

CRISPR/Cas9 Cell Lines

Gene targeting of ESR1 in T47D cells was carried out using CRISPR/Cas9 genome-editing as previously described [21]. Briefly, sgRNAs were selected for CRISPR/Cas9 editing of T47D cells in the sequence regions flanking the Y537S and D538G mutations. The sgRNA guide oligonucleotides were subsequently cloned into the PX458 (Addgene) vector that also includes coding regions for Cas9, tracrRNA, and GFP expression. This plasmid was co-transfected in addition to the appropriate double stranded oligonucleotides into T47D cells. Transfected, GFP-positive cells were sorted *via* FACS analysis, and specific ESR1 mutations were confirmed by sequencing and digital droplet PCR [5]. All T47D cell lines were grown in RPMI-1640 media supplemented with 10% FBS (Corning), 1% Penicillin-Streptomycin (Life Technologies), and 1% L-Glutamine (Life Technologies), and were maintained in a 37°C and 5% CO₂ incubator. Lentiviral T47D cell lines were grown in identical media supplemented 0.5 µg/mL puromycin (Gibco).

Lentiviral Stable Cell lines

ESR1 plasmids containing either wild type (WT) or mutant (Y537S; D538G) variants containing an HA (hemagglutinin) epitope tag, a fluorescent (mCherry) marker for lentiviral transduction, and an antibiotic selection marker (puromycin) were generated by GeneCopoeia™ using the Lenti-Pac™ HIV expression packaging kit for production of recombinant lentiviruses. Briefly, the HA-tagged ESR1 open reading frames in a pcDNA3.1 backbone vector were used for PCR amplification and sub-cloning using EcoR1 and EcoRV restriction sites into the lentiviral transfer vector using the following primer pair: (forward): 5′ – caccatggcatatccatgatgacgtcccagactatgcc – 3′ and (reverse): 5′ – tcagaccgtggcagggaaaccctct – 3′. The presence of WT, D538G, and Y537S variants of ESR1 were verified by DNA sequencing. Recombinant lentiviral particles were generated and harvested by co-transfecting the ESR1 lentiviral expression plasmid together with the LentiPac HIV Packaging plasmid kit into GeneCopoeia 293Ta lentiviral packaging cells. T47D cells were plated at a density of 10×10^4 cells per well in a 24-well plate. For each well, 0.5 ml of suspension, containing the pseudovirus particles at varying MOIs (0.5, 1, 2, 5, and 10), was diluted in complete media with Polybrene transfection reagent (final concentration of 5–8 µg/ml). Multiple stable expressing cell lines were selected for and isolated for further characterization. Individual cell lines were maintained in RPMI-1640 media supplemented with 10% FBS (Corning), 1% Penicillin-Streptomycin (Life Technologies), 1% L-Glutamine (Life Technologies), and supplemented with 0.5 µg/mL puromycin (Gibco) for selection. Cells were maintained in a 37°C and 5% CO₂ incubator.

Transactivation Assay

To begin estrogen deprivation, 4×10^6 cells were transferred from complete media into a T75 flask (Corning) containing Assay Media consisting of phenol-free Improved Minimal Essential Medium (IMEM; Life Technologies) supplemented with 5% Charcoal Stripped Serum (Life Technologies) and 1% Penicillin-Streptomycin (Fisher Scientific). The following day, cells were trypsinized (Life Technologies) and plated into a 96-well flat-bottom opaque plate at a density of 6000 cells per well using a Multidrop™ Combi Reagent Dispenser (Fisher Scientific). Cells were grown for 24 hours and then transfected at 75 ng/

well with either 3X ERE-TATA or pRL-SV40 reporter plasmid using Opti-MEM (Life Technologies) and X-tremeGene HP according to manufacturer's protocol and incubated for 18 hours overnight. The ratio of DNA:X-tremeGene HP used was 1 µg DNA:3 µl X-tremeGene. The following day, transfection media was replaced with fresh Assay Media containing the appropriate inhibitor. The following compounds in these studies were used: 4-OHT, fulvestrant, AZD9496, or peruvoside. The indicated inhibitor was added to cells at the appropriate concentration range and incubated for 6 hours prior to the addition of 20 pM E2 or vehicle control. The final concentration in each well of ethanol and DMSO was 0.125% (v/v) and 0.1% (v/v), respectively. Cells were then incubated overnight and media was changed the next day immediately before detection of luciferase activity. Luciferase activity (luminescence) for triplicate wells was detected with the EnVision Multilabel Plate Reader (Perkin Elmer) using the One-Glo Reporter Assay System (Promega) according to the manufacturer's protocol. The percent inhibition of ER transactivation was calculated by normalizing the luciferase activity with the minimum and maximum controls on each plate. Individual IC₅₀ values for the tested inhibitors were calculated using the variable slope (4 parameter) sigmoidal dose-response model (GraphPad Prism 7 statistical software) [24]:

$$\%Inhibition=y=A+\frac{B-A}{1+(C/X)^D}$$

Here, y represents the percent inhibition and X was the corresponding inhibitor concentration. The fitted C parameter was the IC₅₀ and defined as the concentration giving a response halfway between the fitted maximum (B) and minimum (A) of the curve. For these studies, the maximum was defined as the luciferase value for cells treated with 20 pM E2 in the absence of E2 and the minimum was defined as the luciferase value for WT cells treated without E2 or inhibitor. For each cell line tested, > 3 independent trials with triplicate data points were performed to generate P-values using an unpaired, 2-tailed t-test with Welch's correction to compare WT IC₅₀ values to mutant Y537S and D538G cell lines. Based upon initial studies employing dose ranges spanning 5 orders of magnitude, the following 8-point (3-fold dilution) overlapping dose ranges were used to determine inhibitor IC₅₀ values:

CRISPR-edited cell lines:

Parental and Wild Type (WT): fulvestrant at 0-81 nM; 4-OHT at 0-324 nM; peruvoside at 0-1620 nM; AZD 9496 at 0-12.8 nM.

Y537S and D538G Mutants: fulvestrant at 0-243 nM; 4-OHT at 0-972 nM; peruvoside at 0-1620 nM; AZD 9496 at 0-162 nM.

Lentiviral cell lines:

Wild Type (WT): fulvestrant at 0-81 nM; 4-OHT at 0-324 nM; peruvoside at 0-1620 nM; AZD 9496 at 0-162 nM.

Y537S and D538G Mutants: fulvestrant at 0-243 nM; 4-OHT at 0-972 nM; peruvoside at 0-1620 nM; AZD 9496 at 0-486 nM.

Estrogen Dose-Response

Cells were cultured, plated, and transfected as described for the transactivation assay. The following day after transfection with 3X-ERE, cells were treated with a 10-point (3-fold) E2 dose range from 0 - 1 nM and incubated overnight. Luciferase activity (luminescence) was detected for triplicate wells using the One-Glo Reporter Assay System (Promega) as described above. The percent inhibition of ER transactivation was calculated by normalizing the luciferase activity with the minimum and maximum controls on each plate. Individual EC₅₀ values for the tested inhibitors were calculated using the variable slope (4 parameter) sigmoidal dose-response model (GraphPad Prism 7 statistical software) equation described for the inhibitor studies. For each cell line tested, > 3 independent trials with triplicate data points were performed to generate P-values using an unpaired, 2-tailed t-test with Welch's correction to compare WT and mutant EC₅₀ values.

Immunoblot Analysis

Cells were plated at a density of 8×10^5 in 6 well plates (Costar) in Assay Media for 3 days to deplete estrogen. Cells were then treated with 0, 20, or 200 pM E2 and incubated overnight. Cells were then solubilized for 15 min. on ice in 0.5 mL RIPA buffer (Fisher Scientific) containing 1X Halt™ protease/phosphatase inhibitor cocktail (Fisher Scientific). Supernatant from centrifuged samples was collected and total protein concentration for each sample was determined using a Bradford protein assay (BioRad). 30 µg of total protein from each sample was combined with Laemmli sample buffer containing % 2-mercaptoethanol (Sigma) and heated to 100°C for 10 min. and loaded onto a 4–20% Tris-Glycine mini-gels (Fisher Scientific). The denatured samples were electrophoresed and transferred to nitrocellulose membranes (Fisher Scientific). Membranes were then blocked and incubated overnight in the appropriate primary antibody solution (1:1000 dilution): anti-ERα (rabbit mAb; 8H8; Cell Signaling Technologies), anti-β-actin (rabbit mAb; D6A8; Cell Signaling Technologies) and anti-phospho- ERα(Ser118) (mouse mAb; I6J4; Cell Signaling Technologies). Membranes were then washed and incubated for 1 h with species-appropriate HRP-conjugated secondary antibodies (1:3000). HRP reactive bands were detected using Clarity Western ECL substrate (BioRad). The relative molecular mass of immunoreactive bands was determined using MagicMark™ Western Protein Standards (Life Technologies). Gel images were captured using a Fujifilm LAS 3000 Luminescent Image Analyzer and unedited .tif image files were imported into ImageJ to perform relative gel band densitometry quantification as previously described [25]. Briefly, after individual bands in each gel lane were selected, individual band intensities were calculated by analyzing the total area under the histogram for each lane of the gel. Each histogram peak was analyzed at the same distance from its baseline in order to subtract background equally from each gel lane. For lysates prepared from lentiviral cells, ER was detected as a single band using anti-ERα (rabbit mAb; 8H8) from Cell Signaling Technologies; however, ER was detected as a doublet using anti-ERα (mouse mAb; 6F11) from Leica Biosystems.

Results

Individual ESR1 mutations confer distinct estrogen response phenotypes

We first examined estradiol (E2)-dependent transactivation in parental and CRISPR/Cas9 genome-edited cell lines using an ERE-luciferase-based assay (as outlined in Figure S1). A 10-point E2 dose-response study was performed on two individual and representative WT and mutant (Y537S and D538G) cell lines as well as the parental T47D cell line (Figure 1A). In contrast to the parental and WT controls, both the Y537S and D538G-expressing clones exhibited constitutive (i.e. E2-ligand depleted) ERE-dependent transactivation (Figure 1B). The levels of constitutive activity for both mutant-expressing clones were comparable to the levels observed in parental and WT clones stimulated with maximum E2 (1nM) (Figure 1A), although in three independent experiments the Y537S clones displayed significantly higher (35%) constitutive activity than the D538G clones. (Figure 1B). This quantitative characterization of Y537S and D538G ER constitutive activity is both consistent and complementary to previous studies employing transient overexpression of ESR1 mutants [6, 8-10, 12-15, 26] and to very recent studies involving genome-edited cell lines [21, 27, 28]. Interestingly, we found that the Y537S and D538G mutant-expressing clones exhibit distinct E2-dependent phenotypes. Whereas the activity of the Y537S clones is relatively E2-insensitive the D538G-expressing clones show a 4-5 fold increase in E2-dependent transactivation above the constitutive activity. (Figure 1A and C). The EC₅₀ (half maximal effective concentration) values for E2 stimulation of both the WT and D538G mutant clones is 20 pM (Table S1), consistent with both physiologically relevant concentrations of circulating E2 and with EC₅₀ values obtained in other studies using parental T47D cells [29-31]. Next, a parallel set of studies was performed employing multiple stable lentiviral-transduced clones comparably overexpressing HA-tagged wild type and Y537S and D538G mutant ER at levels 2-3 -fold higher than those in the parental T47D cell line (Figure S3). Very similar E2- dependent and -independent phenotypes to those obtained with the CRISPR/Cas9 edited cell lines were observed (Figure S2). The Y537S lentiviral clones displayed significantly higher (30%) constitutive activity than the D538G-expressing clones (Figure S2A and B). While the constitutive activity of the Y537S clones remained invariant in the presence of E2, D538G mutants displayed a 4-fold enhancement of its constitutive transactivation.(Figure S2A and C) in the presence of E2. These studies suggest that the distinct phenotypes conferred by ER mutants do not result from non-specific stochastic events introduced by either the gene editing or overexpression experimental procedures.

To corroborate these distinct phenotypes conferred by mutant ESR1 in an entirely endogenous system, we determined the state of wild type and mutant ER activation by measuring the level of Serine118 [ser(118)] phosphorylation using quantitative immuno-blot analysis (Figure 2). Serine118 phosphorylation has been previously demonstrated to be required for the full activation of ER activity [32-34]. In contrast to both the parental and wild type controls, both Y537S- and D538G-expressing mutant cell lines exhibited constitutive ser(118) phosphorylation (Figure 2A and 2B). As seen with the ERE-transactivation assay, the levels of constitutive ser(118) phosphorylation of both mutant-expressing clones were comparable to the levels observed in E2-stimulated wild type and parental controls (Figure 2B; Table S2). Analogous to our studies described above using the

transactivation assay, the E2-dependency for the ser(118) phosphorylation phenotype is distinct between the Y537S and D538G mutant-expressing clones (Figure 2). The Y537S clones are relatively E2-insensitive, as no appreciable difference is observed between constitutive and E2-stimulated ser(118) phosphorylation. The D538G-clones, however, show a 3-fold increase in E2-dependent ser(118) phosphorylation (Figure 2B; Table S2). We also examined wild type and mutant E2-dependent ER activation by measuring ser(118) phosphorylation using quantitative immunoblot analysis in the stable lentiviral-transduced cells (Figures S3). Similar to our findings in CRISPR-edited cells, both the Y537S and D538G lentiviral-expressed ER mutants exhibited constitutive ser(118) phosphorylation compared to both parental and WT controls. (Figure S3A and B). As was the case in the CRISPR/cas9-edited system, the E2-dependency for ser(118) phosphorylation of the mutant ERs demonstrated distinct profiles. The constitutive Ser(118) phosphorylation of Y537S ER was E2-insensitive, while for the D538G ER its constitutive phosphorylation was enhanced 2.5-fold when treated with 20 pM E2 (Figure S3A and B). The main difference between ER ser(118) phosphorylation profiles in the genome-edited and overexpression systems is the relatively high level of constitutive phosphorylation observed in Y537S mutants compared to D538G (Figure S3B). Normalization to the total amount of ER expressed indicates that this observed difference is due to an increase in the percentage of phosphorylated Y537S and is not due to differences in ER expression (Table S2). As indicated and described in detail (Figure S3A), in addition to the detection of the full-length protein, we also observed both higher and lower molecular weight ER-specific bands in the ER overexpressing cells that were not present in the parental control; however, none of these immunoreactive species demonstrated constitutive or E2-dependent ser(118) phosphorylation. This suggested that they were not functionally relevant forms of ER and were not further characterized. These results using corroborative ERE-dependent transactivation reporter and quantitative endogenous phospho-immunoblot assays in both CRISPR/cas9 gene-editing and lentiviral induced-overexpression systems show that Y537S and D538G ER confer distinct phenotypes. While both confer a constitutive activity, the constitutive activity of D538G can be enhanced in the presence of physiological levels of estrogen.

ESR1 mutants confer resistance to ER antagonists that can be overcome by higher concentrations of inhibitor

Previous studies, based primarily on transient expression of mutant ESR1 [9, 12] and also recent studies using genome-edited T47D cells published during the course of these studies [21, 26, 28] have suggested that these LBD mutations can confer resistance to ER antagonists. In addition, retrospective analysis correlating patient outcomes with these mutations suggest that this resistance may be clinically relevant [5, 16, 35, 36]. Therefore, we quantified the level of resistance to fulvestrant, 4-OH-tamoxifen (4-OHT) and AZD 9496 conferred by each of the two ESR1 CRISPR mutants using our ERE-dependent luciferase assay conducted initially in the presence of physiological E2 concentration (20 pM). Expression of either mutation, each from two independent clones resulted in significantly increased IC₅₀ (half maximal inhibition concentration) values for all three drugs compared to WT and parental controls (Figure 3A–C; Table S3). The expression of Y537S conferred an approximate and significant 10-fold increase in IC₅₀ value for fulvestrant, 4-OHT, and AZD 9496, while a significant 5-fold increase in IC₅₀ value for the tested inhibitors was

consistently observed in D538G-expressing cells. Despite this rightward shift in IC₅₀ values, 100% blockage of ER transactivation could be achieved in each case at higher doses of drug without an appreciable change in Hill slope (Figure 3A–C; Table S3). Analogous studies conducted under estrogen-depleted conditions demonstrated that the intrinsic constitutive transactivation of the mutant ESR1 expressing cells could be completely blocked by these drugs with IC₅₀ values indistinguishable from those determined in the presence of 20 pM E2 (Table 1; Figure S4A–C). Additionally, we observed comparable phenotypes in the lentiviral-transduced Y537S and D538G clones when examining their resistance profiles to these ER antagonists, further corroborating the findings in CRISPR/Cas9 edited cells (Figures S5A–C; Table S4).

Regarding the ability of high dose fulvestrant, 4-OHT, and AZD 9496 to overcome the resistance conferred by ESR1 mutations, the aforementioned studies *per se* do not rule out the possibility that confounding ERE (i.e., estrogen signaling)-independent mechanisms contribute to this observation. Therefore, we studied the ability of these ER antagonists to block luciferase expression driven by ERE-independent promoters and confirmed their specificity (Figure S6A–C). Conversely, the cardiac glycoside, peruvoside with a canonical mode-of-action distinct from ER signaling [37–39], equipotently blocks luciferase expression driven by either an ERE-dependent or ERE-independent promoter (Figure S6D). Accordingly, resistance to ER-specific ligands conferred by ESR1 mutations is not evident for peruvoside (Figures 3D, S4D, S5D). The demonstration here that these antagonists retain their specificity for ER blockade at levels sufficient to overcome resistance has potential implications for the use of higher doses in the clinic provided these doses are safe and, from a pharmacokinetic/pharmacodynamic (PK/PD) perspective, attainable.

Discussion

A large body of genetic, biochemical and clinical evidence strongly support the hypothesis that ESR1 mutations (e.g., D538G, Y537S) are selected for during estrogen deprivation therapy (i.e., aromatase inhibition) and, as a result of the ligand-independent signaling activity that these mutations engender, drive metastatic disease progression [7, 11, 18, 40, 41]. We and others have observed polyclonality of ESR1 mutations in 5–20% of patients that likely represents convergent evolution wherein multiple metastatic subclones acquire independent activating ESR1 mutations to confer a similar phenotype (i.e., constitutive ER signaling) [5, 16–20, 42, 43]. Interestingly however, longitudinal analyses of cfDNA in patients with polyclonal ESR1 mutations also revealed that individual ESR1 mutations responded differently during treatment [5, 42, 43]. Therefore, this study was designed to identify possible differences between the phenotypes conferred by D538G and Y537S with the objective that this knowledge could optimize the impact of using cell-free DNA molecular analysis as a predictive marker of disease progression and resistance to therapy.

Key observations of our present study indicate that in contrast to the Y537S mutation, the D538G mutation preserves responsiveness to physiological levels of estrogen resulting in a distinctively enhanced ER activation phenotype. Thus, in patients who may have had only a partial lowering of estrogen levels during aromatase inhibitor treatment or in patients following termination of estrogen deprivation therapy rendered less effective due to the

constitutive ER activation of either mutant, the D538G expressing clones would be expected to be in an enhanced ER signaling state in comparison to wild type and the estrogen-independent Y537S expressing clones. Evidence in support of this hypothesis comes from the ESR1 mutation-driven subgroup analysis for progression free survival (PFS) in the exemestane arm of the phase III BOLERO-2 study [42]. Patients with a D538G mutation had a shorter PFS than patients expressing wild type or Y537S ESR1. Larger prospective trials in which patients are stratified on the basis of individual ESR1 mutations that can be measured longitudinally in cfDNA are needed to confirm that the distinct phenotypes we have observed between D538G and Y537S can impact clinical outcome beyond the shared constitutive ER signaling phenotype *per se*. Nevertheless, our current study provides a mechanistic hypothesis for how D538G ESR1 expressing clones can contribute to shorter PFS even in patients such as those in the phase III Bolero-2 study with advanced metastatic disease who have previously experienced several lines of therapy that likely have engendered multiple mechanisms of resistance. Thus, in those patients with dominant D538G-expressing clones, longitudinal analysis for this mutation in cfDNA may prove beneficial for informing more optimal therapeutic regimens involving the continuation of “highly active” estrogen deprivation therapy in combination with palbociclib, fulvestrant, and next generation SERDs.

Previous *in vitro* studies have suggested that Y537S and D538G engender partial resistance to SERMs and SERDs that can be overcome at super-pharmacologic doses [6, 8–10, 12–14, 21, 26, 28, 41]. Such resistance to these anti-endocrine therapies could potentially compromise the treatment of patients having ESR1 mutations who accordingly, have failed aromatase inhibition. In this study, using complete and quantitative dose response analysis, we confirmed these observations in CRISPR/cas9 genome edited ER-dependent cell lines and corroborated these analyses in corresponding lentiviral transduced stable parental cell lines. Furthermore, we demonstrated using pharmacologic and genetic-based controls for off-target effects that the high doses of SERMs and SERDs used to overcome this resistance were acting through an ER-specific mechanism in these model systems. Given the potential significance of these *in vitro* studies, we considered these findings in the context of the FERGI trial [44] to help assess their clinical relevance. In this phase II study, baseline plasma samples of patients who had progressed on aromatase inhibitor treatment were randomized either to fulvestrant combined with a pan-PI3K inhibitor pictilisib or to the combination of fulvestrant and placebo and were examined for ESR1 and PIK3CA mutations in tissues and cfDNA. Clinical analysis of both fulvestrant treatment arms of the trial indicated that patients with ESR1 mutations in circulating tumor DNA (ctDNA) had no diminished PFS in either arm compared to patients expressing only ESR1 wild type [43]. In the PALOMA-3 study [45], patients who failed on prior endocrine therapy were randomized to fulvestrant in combination with the CD4/6-inhibitor palbociclib or to fulvestrant and placebo. Consistent with the FERGI trial, the PFS benefit was not significantly diminished in those patients expressing ESR1 mutations compared to ESR1 wild type in either treatment arm although median PFS appeared slightly worse in the ESR1 mutated patients treated with fulvestrant alone [46]. Thus, from the evidence obtained in these two particular trials, it appears that the partial resistance to fulvestrant engendered by ESR1 mutations *in vitro* may not translate into a clinically relevant finding.

It has been suggested that one explanation for this apparent discrepancy may result from the design of several previous studies in which reduced sensitivity of Y537S and D538G mutant ER to fulvestrant was observed in ER-negative cell line models where mutant ER homodimers may behave differently from the physiologically relevant mutant/wild type ER heterodimers [12, 41, 43]. Importantly, in this current study, the genome edited ER⁺ cell models used have been documented to be heterozygous for each mutant ESR1 allele [21] and the observed mutant ER-dependent resistance to fulvestrant was quantitatively corroborated in stable lentivirus transduced mutant ER cell lines derived from parental cell lines endogenously expressing wild type ER. Therefore, based on this study and other recent reports [9, 12, 21, 26–28], an alternative explanation for the apparent discrepancy between the observed *in vitro* resistance to fulvestrant and the lack of diminished PFS in those patients with mutant ER-expressing metastases should be considered.

In this regard, patients in the FERGI trial that had radiographically confirmed responses, either complete (CR) or partial (PR), showed significant decreases in both plasma PIK3CA and ESR1 variant allele frequencies (VAF) that were apparent at the earliest times assessed [43]. However, decreases in both plasma PIK3CA and ESR1 VAFs of similar magnitude were also observed in a substantial fraction of patients whose best response was either stable disease or progressive disease. This latter observation raises the possibility that disease progression was due to growth of ESR1 wild type lesions that harbor mutations in alternative genes that were not being tracked in this study. Therefore, the decreases in ESR1 VAF seen in patients with stable or progressive disease are not uniquely consistent with fulvestrant directly down-modulating ER signaling in mutant ESR1 clones. Alternatively, the outgrowth of wild type ESR1 clones driving disease progression could be selected, particularly in the presence of estrogen where in this setting mutant ESR1-expressing clones might even be deselected and together result in lower ESR1 VAFs. Increases in ESR1 VAF were also observed, and as might be expected, only in the cfDNA of patients with stable or progressive disease [43]. Given the low (0.45%) median ESR1 VAF, a treatment protocol that did not involve estrogen deprivation therapy, and a complex association of plasma ESR1 VAF with clinical response to fulvestrant, it is difficult to discern differential clinical responses to specific treatments of patients with mutant ESR1-expressing tumors compared to those with wild type. Larger prospective trials in which patients are stratified on the basis of individual ESR1 mutations that can be measured longitudinally in cfDNA are needed to determine if fulvestrant does have reduced activity in patients with ESR1 mutations and, if so whether this difference can be overcome with next generation SERDs that possess more favorable pharmacodynamic profiles than fulvestrant. An important component of the design of such studies, in contrast to the FERGI trial, would be combination therapy with mainstay estrogen deprivation to help ensure that mutant ESR1 clones, selected (through their ligand-independent ER signaling activity) among heterogeneous wild type subclones, are dominant drivers of disease progression.

The complex association of plasma ESR1 VAF with clinical response to fulvestrant in the aforementioned FERGI trial may result not only from clonal heterogeneity *per se* but also from intercellular interactions within the tumor microenvironment that may support distinct metastatic phenotypes [47, 48]. Thus, differences in the intrinsic response of distinct clones to fulvestrant for example, may be modulated by their individual microenvironments (e.g,

different metastatic sites within the same patient). Therefore, a limitation of this in vitro study is its exclusive use of cell autonomous conditions. Using the cell lines described here, studies in microphysiological systems that recapitulate critical aspects of the breast cancer metastatic niche have been initiated aimed at identifying emergent ER-dependent heterotypic signaling networks that may represent targetable metastatic dependencies for mutant ESR1 expressing clones [49, 50]. Moreover, a better understanding of LBD mutant dependencies in the metastatic setting will guide the design and development of novel therapeutics that specifically target mutant ESR1 expressing clones. As described above, the ability of high doses of antagonists to overcome the resistance conferred by ESR1 mutations specifically through an on-target mechanism has prompted interest in the development of next generation ER antagonists with improved efficacy for mutant ER. One example of this is the SERD AZD 9496, which has been shown to reduce the expression of clinically-relevant ESR1 mutations in vitro and reduce tumor growth in D538G patient-derived xenograft in vivo models [26, 51]. We expect that the design and development of these next generation antagonists will be greatly facilitated by the proactive implementation of complementary approaches that can anticipate resistance mechanisms involving either secondary mutations in ER itself or networks that can bypass ER signaling dependencies [52–55]. Given the high selection pressure and low barrier to evolve resistance to endocrine therapy through mutations in the LBD and the identification of constitutively active ER structural variants devoid of an LBD [14], the development of next generation ER antagonists will also likely include those that interact outside the LBD.

In summary, while there have been many studies focused on the constitutive ER signaling activity commonly engendered by ligand binding domain mutations selected during estrogen deprivation therapy, comparatively few have investigated distinctive phenotypes conferred by different mutations within this class. Key observations with the two most commonly observed mutations in the patients indicate that in contrast to the Y537S mutation, the D538G mutation preserves responsiveness to physiological levels of estrogen resulting in a distinctively enhanced ER activation phenotype compared to the wild type and estrogen-independent Y537S expressing clones. These observations provide a tenable hypothesis for how D538G ESR1 expressing clones can contribute to shorter PFS even in patients such as those in the phase III BOLERO-2 study with advanced metastatic disease who have previously experienced several lines of therapy that likely have engendered multiple mechanisms of resistance. Thus, in those patients with dominant D538G-expressing clones, longitudinal analysis for this mutation in cfDNA may prove beneficial for informing more optimal therapeutic regimens involving the continuation of “highly active” (a term borrowed from the durable treatment of HIV resistance) estrogen deprivation therapy in combination with palbociclib, fulvestrant, and next generation SERDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ESR1; ER	estrogen receptor alpha
LBD	ligand binding domain
cfDNA	cell-free DNA
CRISPR	clustered regularly interspersed short palindromic repeats
ERE	estrogen response element
SERD	selective estrogen receptor degrader
SERM	selective estrogen receptor modulator
E2	estradiol
ser118	Serine 118
PK/PD	pharmacokinetic/pharmacodynamic
PFS	progression free survival
ctDNA	circulating tumor DNA
CR	complete response
PR	partial response
VAF	variant allele frequency

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Fig 1.

ESR1 mutations confer distinct estrogen response phenotypes in CRISPR-edited cells. (A) Parental (green), wild-type (WT; gold), D538G (blue), and Y537S (red) cells were deprived of E2 for 3 days and then treated with a 10-point E2 dose range (0–1 nM). Both Y537S and D538G mutants show increased constitutive activity that is comparable to the maximal estrogen-induced activity of the control parental and WT lines. D538G mutants also display enhanced maximal E2-dependent activity. E2 EC₅₀ values for control and D538G cells are comparable and are within the physiological range (20 pM; see table S1). In this representative graph, the mean $\pm$ SEM of triplicate data points is plotted in panel A. Independent experimental trials were repeated 3 times. (B and C) Quantification of luciferase activity demonstrates differences in constitutive transactivation (B) and maximal E2-dependent transactivation between Y537S and D538G mutants relative to their respective constitutive activity (C). Both Y537S and D538G mutant clones display significantly higher

constitutive transactivation compared to WT controls in the absence of E2. However, Y537S expressing cells also have significantly higher constitutive activity compared to that of D538 mutants (B). In addition, while both WT and D538G expressing cells show increased E2-dependent transactivation relative to their constitutive activities, Y537S mutants do not demonstrate any E2-dependent transactivation (C). P-values were obtained from the results of 3 independent experiments ($n = 3$) with triplicate data points using an unpaired, 2-tailed t-test with Welch's correction; * = $p < 0.05$; ** = $p < 0.005$.

Fig 2.

ESR1 mutations display distinct ser-118 phosphorylation phenotypes in CRISPR-edited cells. (A and B) 30 μ g of total protein was loaded per lane from extracts prepared from parental, WT, Y537S, or D538G cells grown in the absence or presence of 20 pM E2. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for pER(Ser118), ER and actin (arrowheads). In the absence of E2 (blue bars), significant constitutive ER phosphorylation was detected in both Y537S and D538G mutants, and no significant phosphorylation above background (see Methods) was detected in parental or WT controls (blue asterisks; ** = $p < 0.005$). For cells cultured in the presence of 20 pM E2 (red bars), a significant increase in ER phosphorylation is observed in both WT and D538G clones compared to their respective constitutive pER levels (red asterisks; * = $p < 0.05$; ** = $p < 0.005$), while no E2-dependent ser(118) phosphorylation is observed in Y537S clones. The gel image shown is a representative result from 3 independent trials conducted using freshly prepared cell lysate. Gel band intensity for each pER α -specific band was quantified using ImageJ densitometry software and normalized to the total amount

of actin in each lane. The graph summarizes the average pER band intensities calculated from 3 independent experiments ($n = 3$), $\pm$ SEM. P-values were obtained using an unpaired, 2-tailed t-test with Welch's correction.

Fig 3.

ESR1 mutations confer partial resistance to ER antagonist treatment in CRISPR-edited cells. Representative dose-response curves for parental (green), WT (gold), Y537S (red), and D538G (blue) cell lines treated with an 8-point dose response of fulvestrant (A), 4-OHT (B), AZD 9496 (C), or (D). Cells were deprived of E2 for 3 days, transfected with ERE plasmid overnight, and 6 h after inhibitor addition the following day, were treated with vehicle or 20 pM E2 (see Figure S1). After 24 h, luciferase activity was detected as an indicator of ERE-dependent transactivation. Statistical summary of IC₅₀ values for CRISPR-edited cells

treated with inhibitors shows that both Y537S and D538G mutants have significantly increased IC_{50} values compared to parental/WT controls (red asterisk) when treated with fulvestrant (A; 10-fold and 5-fold), 4-OHT (B; 6-fold and 3-fold), or AZD 9496 (C; 10-fold and 5-fold). The IC_{50} values of inhibitor-treated Y537S mutants are significantly higher than those of D538G mutants (black asterisk). Resistance in both mutants was able to be overcome and reach 100% inhibition at higher inhibitor concentrations. No apparent resistance was observed for the control peruvoside-treated mutants (D; see Fig S6 for description of peruvoside control experiment). For each cell line tested, the average luciferase values over the inhibitor dose range were used to determine individual IC_{50} values $\pm$ SEM. P-values (* <0.05 ; ** <0.005 ; *** <0.0005 ; **** <0.00005) were obtained using an unpaired, 2-tailed t-test with Welch's correction comparing WT IC_{50} values to those of Y537S-2 and D538G mutants. For each cell line tested, 5 independent experiments with triplicate data points were conducted. A complete summary of statistical analysis, including Hill slopes and number of experimental replicates of these studies is presented in Table S3.

Table 1

ESR1 mutations confer similar resistance phenotypes to ER antagonists in the presence or absence of E2.

Cell Line:	Parental		WT-1		WT-2		Y537S-1		Y537S-2		D538G-1		D538G-2	
E2:	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Fulvestrant: (nM) ± SEM		0.9 ± 0.2		1.2 ± 0.2		1.0 ± 0.2	11.0 ± 1.8	9.1 ± 0.9	14.3 ± 0.9	11.9 ± 1.5	5.1 ± 0.6	5.3 ± 0.6	4.3 ± 0.8	4.9 ± 0.8
4-OHT: (nM) ± SEM		7.0 ± 0.2		10.0 ± 1.6		7.1 ± 0.3	68.3 ± 7.4	64.6 ± 1.6	63.2 ± 6.5	60.4 ± 3.7	32.6 ± 1.1	32.0 ± 2.5	38.1 ± 4.0	37.4 ± 5.9
AZD 9496: (nM) ± SEM		1.0 ± 0.1		0.6 ± 0.1		0.6 ± 0.2	10.1 ± 1.4	12.3 ± 1.3	10.6 ± 0.7	12.2 ± 1.0	8.3 ± 2.0	7.0 ± 0.7	6.0 ± 0.6	5.7 ± 0.2
Peruvoside: (nM) ± SEM	47.5 ± 5.0	46.7 ± 4.3	49.1 ± 3.7	48.3 ± 2.5	49.4 ± 7.8	53.3 ± 0.2	42.1 ± 6.6	41.9 ± 4.2	44.9 ± 7.0	47.4 ± 5.9	47.3 ± 1.5	43.5 ± 0.7	41.4 ± 2.3	43.3 ± 3.9

Parental, WT, Y537S or D538G cells were transfected with an ERE-luciferase reporter construct in hormone-depleted media and were treated with fulvestrant, 4-OHT, AZD 9496, or peruvoside and incubated either in the presence or absence of E2. Mean luciferase values for each cell line were used to determine individual IC₅₀ values under each conditions of E2 depletion (light gray) or stimulation with 20 pM E2 (dark gray). In the absence of E2, no dose-response was observed for parental/WT cells treated with fulvestrant, 4-OHT, or AZD 9496. However, an E2-independent dose-response was observed for parental/WT cells treated with peruvoside (see Fig S6 for description of peruvoside control experiment). For each cell line tested, 3 independent experiments with triplicate data points were conducted. No significant difference in IC₅₀ value was observed for either Y537S or D538G mutants in the presence or absence of E2. IC₅₀ values obtained in these studies for E2-stimulated cells were similar in magnitude to those reported in Table S3. Representative dose-response curves for these studies are shown in Fig S4.