Priority Report

D538G Mutation in Estrogen Receptor-α: A Novel Mechanism for Acquired Endocrine Resistance in Breast Cancer

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Abstract

Resistance to endocrine therapy occurs in virtually all patients with estrogen receptor α (ERα)-positive metastatic breast cancer, and is attributed to various mechanisms including loss of ERα expression, altered activity of coregulators, and cross-talk between the ERα and growth factor signaling pathways. To our knowledge, acquired mutations of the ERα have not been described as mediating endocrine resistance. Samples of 13 patients with metastatic breast cancer were analyzed for mutations in cancer-related genes. In five patients who developed resistance to hormonal therapy, a mutation of A to G at position 1,613 of ERα, resulting in a substitution of aspartic acid at position 538 to glycine (D538G), was identified in liver metastases. Importantly, the mutation was not detected in the primary tumors obtained prior to endocrine treatment. Structural modeling indicated that D538G substitution leads to a conformational change in the ligand-binding domain, which mimics the conformation of activated ligand-bound receptor and alters binding of tamoxifen. Indeed, experiments in breast cancer cells indicated constitutive, ligand-independent transcriptional activity of the D538G receptor, and overexpression of it enhanced proliferation and conferred resistance to tamoxifen. These data indicate a novel mechanism of acquired endocrine resistance in breast cancer. Further studies are needed to assess the frequency of D538G-ERα among patients with breast cancer and explore ways to inhibit its activity and restore endocrine sensitivity. Cancer Res; 1–9. © 2013 AACR.

Introduction

The human estrogen receptor-α (ERα) belongs to the superfamily of nuclear hormone receptors that function as ligand-activated transcription factors (1). Upon binding of estrogen, the ER dimerizes and binds to coactivators. The complex is then recruited to the estrogen-responsive elements (ERE) on the promoters of ER target genes. Approximately 75% of all breast cancers express ERα, and targeting ERα signaling, either by reducing levels of the ligand or inhibiting the receptor, is a key treatment strategy in these tumors. However, some patients with metastatic breast cancer (MBC) do not respond to any form of endocrine treatment (de novo resistance), and virtually all patients who initially respond eventually develop endocrine resistance (acquired resistance). Mechanisms associated with the development of acquired resistance include reduced expression of ERα, altered activity of coregulators, and increased activity of growth factor signaling pathways (2). Although mutation of key proteins is a common event in tumorigenesis, mutation of ERα is a rare event, occurring in only 1% of primary tumors (3). To our knowledge, acquired mutations of ERα have not been linked to the development of endocrine resistance yet.

We report here on a novel mutation of ERα, in which an A to G substitution at position 1,613 resulted in substitution of aspartic acid at position 538 to glycine (D538G). The mutation was identified in liver metastases obtained from patients who developed endocrine resistance, but not in samples of primary tumors obtained prior to commencing endocrine treatment. Structural modeling indicates that D538G substitution creates a conformational change that disrupts the interaction between the receptor and either estrogen or tamoxifen, but mimics the conformation of the activated receptor. Studies in cell lines confirmed ligand-independent, constitutive activity of the mutated receptor. Taken together, these data indicate the mutation D538G as a novel mechanism conferring acquired endocrine resistance.

Materials and Methods

Patients and genetic analyses

Samples of patients with metastatic breast cancer were submitted, at the discretion of their physicians, for...
commercially available genetic analysis aiming at identifying novel targets for treatment (Foundation One, Foundation Medicine, Cambridge, MA). The test has been described previously (4), and comprises deep sequencing of cancer-related genes on DNA extracted from paraffin-embedded tissue samples. Clinical data was obtained from the treating physicians.

**Chemicals and antibodies**

17β-estradiol (E2) and 4-hydroxytamoxifen (4-HT) were obtained from Sigma. Fulvestrant (ICI 182,780) was obtained from Tocris Bioscience. The antibodies used include anti-ERα (F-10) and anti-p-ERα (Tyr 537), both from Santa Cruz Biotechnology, and anti-SRC-1(MA1-840) from Thermo Fisher Scientific.

**Constructs**

The ERα expression vector (HEGO) was a generous gift of P. Chambon (University of Strasbourg, France). The ERE-luciferase reporter construct, kindly provided by D. Harris, (UCLA, CA), consists of 2 repeats of the upstream region of the vitellogenin ERE promoter.

Generation of pcDNA3 ER-WT and 538G-ERα constructs: full-length ERα was subcloned into EcoRI site of pcDNA3 (Invitrogen) using the primers: 5'-primer: TGGAAATCTCATGACCATGCCTTCACAC, 3'-primer: AAACCTGAGTCAAGCTTGTCAGGGAAA. Generation of 538G-ERα: site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The ERα expression vector was used as a template for PCR. Primers used were as follows: 5'-primer: GTCGCCCTCTATGGCTGGATCTGAGG; 3'-primer: CTCCAGCAGGGATAGGAGGAC. All subcloned constructs were sequenced.

**Cells and transfections**

Cells were grown from the American Type Culture Collection. MCF-7 were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS); MDA-MB-231 were grown in RPMI medium containing 10% FCS. All transfections used Jet Pei (Polyplus Transfection). For all E2, 4-HT, and fulvestrant studies, cells were cultured in phenol-free media using 10% charcoal-treated serum for 2 days before treatment.

**Western blot analysis**

Cells were harvested, lysed, and the total protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/L EDTA, 1 mmol/L NaF). Together with a protease and phosphatase inhibitor cocktails (Sigma), lysates were resolved on 10% SDS-PAGE and immunoblotted with the indicated antibodies.

**Co-immunoprecipitation studies**

Studies were conducted essentially as described previously (5). Cells were harvested in immunoprecipitation (IP) buffer (50 mmol/L Tris–HCl pH 7.5, 1% NP-40, 150 mmol/L NaCl, 5 mmol/L EDTA). Total protein was extracted, and 1 mg protein lysate was incubated with 5 μL of anti SRC-1 antibody overnight at 4°C. Then, protein A/G beads (Pierce) were added and, after a 2-hour incubation, the beads were washed once with IP-buffer and 3 times with PBS. The immunoprecipitated materials were separated by SDS-PAGE and detected by Western blot analysis.

**Luciferase assays**

Cells were plated in 24-well plates and transfected with the reporter vector and the various constructs. Luciferase assay was conducted using the Luciferase Assay System kit (Promega, CA) according to the manufacturer's instructions. Luciferase units were normalized to protein concentration.

**Real-time quantitative PCR**

Two days after transfection with the various constructs, total RNA was prepared using the RNA isolation kit (Sigma). Total RNA (1 μg) was reverse transcribed using RevertAid (Fermentas). The cDNA was then used for real-time quantitative PCR using StepOne Plus (Applied Biosystems). The TGF-α-specific primers used were: 5’CAGGTATGAGTGCAGACC and 3’ACGTACCCGAAGTGGCAGAC. The progesterone receptor (PR)-specific primers used were: 5’CCGCTCTACCTGCGACTC and 3’TGAATCCGGCCTCAGGTT. The GREB1a-specific primers used were: 5’ACGTGTGATGACTGAGTAG and 3’CCAGCCAAGGTAGAGG. Equal loading was determined using β-actin-specific primers.

**Proliferation and migration assays**

Proliferation was assessed using the MTT assay as previously described (5). For the assay, MCF-7 cells were transfected with either a control vector, WT-ERα, or 538G-ERα, plated in 96-well plates (3,000 cells/well), cultured in phenol-free media with 10% charcoal-treated serum for 2 days, and then treated for 48 hours as indicated.

Migration was assessed using the wound healing ("scratch") assay as described previously (6). For the assay, MCF-7 cells were transfected with the indicated constructs and grown to confluence in a six-well plate in phenol-free media with 10% charcoal-treated serum. The cell monolayer was then scraped in a straight line with a 200-μL tip and photographed at 48 hours.

**Structure modeling**

Mutant D538G ERα was modeled twice: (i) according to PDB 20CF (7) for the estrogen-bound conformation, and (ii) PDB 3ERT (8) for the tamoxifen-bound conformation. Both models were created using Jakal as implemented in the Fold and Function Assignment System (FFAS) server (9), and then refined using the Relax protocol of Rosetta version 3.4 (10). On thousand models were generated for each initial model structure and then clustered using the Rosetta cluster protocol. A representative of the largest cluster was selected for further analysis. The wild-type (WT) structure of the estrogen-bound conformation was also modeled based on PDB 20CF.

**Molecular docking**

Molecular docking of estrogen and tamoxifen were performed for both conformations using Dock version 6.5
Results

Identification of D538G mutation in ERα in breast cancer samples

From December 2011 to April 2013, tumor samples of 13 Israeli patients with ERα-positive metastatic infiltrating ductal carcinoma (IDC), who have failed multiple lines of treatment, were submitted for genetic analysis aiming at identifying novel treatment options. Surprisingly, a novel mutation (A1613G) leading to the substitution D538G in ERα was identified in liver metastases obtained from 5 patients (38%). Importantly, the mutation was not detected in the primary tumor of these patients, obtained at diagnosis prior to commencing endocrine treatment. All 5 patients received at least two lines of endocrine treatment for a prolonged duration (from 67 to 97 months of treatment) for the adjuvant and metastatic setting, prior to the development of endocrine resistance (Table 1). The frequency of the mutated allele was 16% to 41% and correlated with the percentage of tumor cells in the samples.

All biopsies containing the mutation were obtained from liver metastases. On the other hand, only one of the eight samples with WT-ERα was obtained from the liver, and the other seven were obtained from bone or soft tissues ($P = 0.005$ for the comparison between the groups). In one patient who carried the mutation, pulmonary metastasis was analyzed simultaneously and did not harbor the mutation. This observation may suggest either emergence of this specific mutation in the liver or predilection of cells harboring the mutation to the liver tissue.

Table 1. Clinical and pathologic characteristics of 5 patients with infiltrating ductal carcinoma carriers of the D538G-ERα mutation

<table>
<thead>
<tr>
<th>Stage at diagnosis</th>
<th>Hormone receptors at primary tumor</th>
<th>ER mutation status at diagnosis</th>
<th>Adjuvant hormonal treatments (months)</th>
<th>Hormonal treatments for metastatic disease (months)</th>
<th>Hormone receptors at metastasis</th>
<th>Tumor content (%)</th>
<th>ER minor allele frequency (mutant/WT, %)</th>
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<tr>
<td>Local</td>
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<td>WT</td>
<td>NA</td>
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<td>Fulvestrant (13)</td>
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<tr>
<td>Local</td>
<td>ER 0</td>
<td>PT</td>
<td>Tamoxifen (60)</td>
<td>Anastrazole (3)</td>
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<td>+1</td>
<td></td>
<td></td>
<td>PR + 3</td>
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<td>Tamoxifen + goserelin (14)</td>
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<td>+3,</td>
<td></td>
<td>Anastrozola + goserelin (14)</td>
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</tbody>
</table>

Abbreviations: ER, estrogen receptor; IDC, infiltrating ductal carcinoma; NA, not available; PT, progesterone receptor; WT, wild-type.

Structural modeling of D538G-ERα

Asp-538 is positioned within a critical area of the ligand-binding domain (LBD), termed helix 12, and in vitro substitution of it to alanine or asparagine reduced the activity of the receptor (12). Aspartic acid contains a fairly large negative side-chain whereas glycine contains only a hydrogen substituent as its side-chain. Thus, the D538G substitution is expected to affect the tertiary structure of ERα. Analysis of the crystal structure of the LBD in the WT-ERα indicates the important role of helix 12 in mediating the interaction between the receptor and various coactivators, including the steroid receptor coactivator (SRC)-1 and -2 (1). Upon binding of estrogen, helix 12 is positioned over the ligand-binding pocket, thus forming a surface for the recruitment of coactivators (Fig. 1A). However, upon binding of tamoxifen, helix 12 is displaced into a position that prevents binding of coactivators (Fig. 1B and 1C). The model structure of D538G-ERα (Fig. 1D) suggests that the conformation of helix 12 in the mutated protein is similar to that observed in the WT-ERα upon estrogen binding (Fig. 1D). Thus, the model predicts ligand-independent constitutive activity of D538G-ERα.

We next compared the effects of D538G substitution to that of Y537S substitution, which also induces constitutive activity of the receptor (13). The model demonstrates a change in side-chain rotamers. The hydrogen bonds Y537-N348 and D351-L540 exist in the WT structure, but the Y537-N348 bond cannot exist in the Y537S mutant (Fig. 1E). Similarly, the D538G substitution prevents the Y537-N348 hydrogen bond but allows the D351-L540 bond to exist (Fig. 1F). These changes in the network of hydrogen bonds may cause higher flexibility in helix 12, thus enabling binding of coactivators even in the absence of a ligand.

We next modeled the docking of estrogen and tamoxifen. The model structure of estrogen- or tamoxifen-bound WT-ERα (based on 2OCF and 3ERT) yielded a similar pose to the one observed in the X-ray structure (1ERE, 2OCF, and 3ERT). The
Figure 1. D538G mutation enables ligand-independent binding of coactivators. A, WT-ERα in the estrogen-bound conformation (PDB 1ERE) is shown by beige ribbons. Helix 12 (H12) colored in brown. E2 of the X-ray structure is shown by yellow VdW spheres, and the docking result is shown by green VdW spheres. B, WT-ERα in the tamoxifen-bound conformation (PDB 3ERT) is shown by blue ribbons. H12 colored in dark blue. Tamoxifen of the X-ray structure is shown by red VdW spheres and the docking result is shown by pink VdW spheres. C, WT-ERα in the estrogen-bound conformation (PDB 1ERE) is shown by beige ribbons. WT-ERα in the tamoxifen-bound conformation (PDB 3ERT) is shown by blue ribbons. The wild-type X-ray structure of Src-1 (PDB 3UUD) is shown by orange ribbons. Estrogen is shown by yellow sticks, and tamoxifen is shown by red sticks. The location of D538 is shown in both conformations by sticks. The direction of H12 in both conformations is shown by a black arrow. SRC-1 cannot bind the tamoxifen-bound receptor because H12 is located in the SRC-1 binding site. D, WT-ERα in the estrogen-bound conformation (PDB 1ERE) is shown by beige ribbons. E2 of the X-ray structure is shown by yellow VdW spheres. The D538G-ERα model structure is shown by dark red ribbons, and the docking result of E2 is shown by green VdW spheres. (Continued on the following page.)
model of D538G-ERα suggests a conformational change in the position of helix 12 that enables binding of coactivators in the absence of estrogen and interferes with binding of estrogen and tamoxifen (Fig. 1G and H). Thus, the D538G receptor is predicted to be resistant to tamoxifen. The molecular docking used here is based on the assumption that the WT protein is rigid. Substitution of Asp by Gly at position 538 allows the protein to be more flexible and may help to understand the low affinity of D538G-ERα to tamoxifen.

**Ligand-independent transcriptional activity of D538G-ERα**

In order to study the activity of the mutated receptor, an ERα harboring the D538G substitution (538G-ERα) was generated and its transcriptional activity was studied compared to WT-ERα using the ERE-luciferase reporter (14). MCF-7 cells were cotransfected with the ERE-luciferase reporter and either pcDNA3, WT-ERα or 538G-ERα. Cells were grown in estrogen-depleted medium and treated with either vehicle control or E2. In the absence of E2, WT-ERα increased ERE activity by 10-fold compared with pcDNA3- and WT-ERα-transfected cells, whereas 538G-ERα increased ERE activity by 45-fold (Fig. 2A, black bars, P = 0.002). Treatment with E2 significantly increased ERE activity only in pcDNA3- and WT-ERα-transfected cells but activity remained lower compared with 538G-ERα-transfected cells (Fig 2A, gray bars). In addition, similar results were noted in the ERα-negative MDA-MB-231 cells. Overexpression of 538G-ERα increased ERE activity by 11-fold compared with pcDNA3-transfected cells (Fig. 2B, P = 0.0002) or WT-ERα–transfected cells (P = 0.0004), and treatment with E2 increased activity in WT-ERα–transfected cells but not in 538G-ERα–transfected cells. In order to further validate the transcriptional activity of 538G-ERα, we also examined its ability to induce transcription of the ERα-regulated genes PR, GREG1, and TGF-α (12). Expression of 538G-ERα in MCF-7 cells increased mRNA levels of PR by 15.7-fold and of GREG1 by 28-fold compared with pcDNA3-transfected cells (Fig. 2C and D), and by 3- and 4-fold, respectively, compared with WT-ERα–transfected cells (P < 0.01 for all comparisons). Similarly, overexpression of 538G-ERα in MDA-MB-231 cells significantly increased TGF-α mRNA levels (Fig. 2E, P = 0.01). Due to the activation of the endogenous ERα, treatment with E2 increased the transcriptional activity in MCF-7 cells transfected with 538G-ERα (Fig. 2C and 2D), but not in MDA-MB-231 cells (Fig. 2E).

Taken together, these data clearly indicate ligand-independent, constitutive activity of D538G-ERα in breast cancer cells.

**D538G-ERα interacts with SRC-1 constitutively in a ligand-independent manner but does not affect phosphorylation of Tyr 537**

Our model predicts that the conformational alteration induced by the substitution of Asp538 to glycine will confer the receptor active conformation allowing ligand-independent interaction with coactivators. In order to test this hypothesis, we examined the interaction between overexpressed WT-ERα or 538G-ERα and endogenous SRC-1 in MDA-MB-231 cells (Fig. 3A). Although WT-ERα interacted with SRC-1 only upon treatment with E2, 538G-ERα interacted with SRC-1 in a ligand-independent manner.

Phosphorylation of Tyr-537 may inhibit activity of ERα, and substitution of it by Asn (Y537N) is associated with increased activity of the receptor (13, 15). We examined the effect of 538G on phosphorylation of Tyr-537, using a phospho–Tyr-537–directed antibody. No change in phosphorylation pattern was noted, suggesting that increased activity of D538G is not mediated by altered phosphorylation of Tyr 537 (Fig. 3B).

**D538G-ERα enhances proliferation and migration of breast cancer cells**

We next tested the ability of 538G-ERα to enhance the proliferation of MCF-7 cells. Cells were transfected with control vector, WT-ERα, or 538G-ERα, grown in estrogen-depleted medium, and treated with either vehicle or E2 for 48 hours. Viability was studied by the MTT assay. The 538G-ERα enhanced proliferation compared with either pcDNA3 or WT-ERα by 33% in untreated cells and by 28% in E2-treated cells (Fig. 4A, P < 0.01 for both comparisons).

In order to study the ability of the mutation to affect migration, we conducted a wound healing (“scratch”) assay. MCF-7 cells were transfected with control vector, WT-ERα, or 538G-ERα, grown in estrogen-depleted medium, and the cell monolayer was scraped in a straight line and then treated with either vehicle or E2 for 48 hours. 538G-ERα enhanced migration compared with either pcDNA3 or WT-ERα in both untreated and treated cells (Fig. 4B).

**D538G-ERα is resistant to tamoxifen and fulvestrant**

We tested the ability of the active metabolite of tamoxifen, 4-HT, and of the selective ER downregulator (SERD) fulvestrant to inhibit the transcriptional activity of 538G-ERα. To this aim, we employed the ER-negative MDA-MB-231 cells, which do not show intrinsic transcriptional activity (Fig. 2B). Cells were transfected with either WT-ERα or 538G-ERα, grown in estrogen-depleted medium and treated with 4-HT (Fig. 5A) or fulvestrant (Fig. 5B) as indicated. The mutated...
receptor demonstrated relative resistance to both 4-HT and fulvestrant.

Discussion

We show here, for the first time, that acquired resistance to hormonal treatment in metastatic breast cancer may be mediated through an activating mutation of ERα. Despite the central role of ERα in the development of breast cancer, mutation of ERα is a rare event (3). Two activating mutations of the ERα have been described previously, Y537N and K303R, and both occur in less than 1% of primary tumors (15–17). To our knowledge, the D538G mutation in ERα has not been described as yet. Furthermore, no other acquired mutations of the ERα are currently known. There are two possible explanations for this. First, prior studies focused mainly on the presence of mutations in the primary tumor at diagnosis and, thus, were not able to detect acquired mutations in the metastases. Second, it is possible that sequencing methods used previously were less sensitive than current methods. We noted the presence of this mutation in 5 out of 13 (38%) patients with ERα-positive metastatic breast cancer. However, these are highly selected, heavily pretreated patients and may not represent the general population of patients with breast cancer. The actual prevalence of the D538G mutation needs to be determined in large cohorts of patients. If indeed the mutation is identified in a significant proportion of patients, direct testing of it may be an easy and cheap method to predict response to hormonal treatment.

Figure 2. 538G-ERα possess constitutive, ligand-independent transcriptional activity. A, MCF-7 cells were transiently transfected with WT-ERα, 538G-ERα, or control vector, together with the ERE-luciferase reporter. Cells were treated with E2 (10 nmol/L) as indicated, for 24 hours. The luciferase activities were analyzed and normalized to total protein concentration, and are shown relative to the control vector. *, P < 0.01, WT-ERα versus control; **, P < 0.005 538G-ERα versus control and WT-ERα. B, MDA-MB-231 cells were transiently transfected and treated as in (A), and luciferase activity was analyzed. *, P < 0.005 WT-ERα versus control; **, P < 0.005 538G-ERα versus control and WT-ERα. C and D, MCF-7 cells were transiently transfected with WT-ERα, 538G-ERα constructs, or a control vector. Cells were then treated with E2 or control vehicle for 24 hours. PR (C) and GREB1a (D) mRNA levels were determined 48 hours after transfection by quantitative RT-PCR. *, P < 0.01 538G-ERα versus WT-ERα. E, MDA-MB-231 cells were transiently transfected and treated as in (C), and TGFα mRNA levels were determined 48 hours after transfection by quantitative RT-PCR. *, P < 0.01 538G-ERα versus WT-ERα. All figures show representative results of at least three independent experiments, each performed in quadruplicates (ERE-luciferase) or triplicates (RT-PCR). Each bar represents the mean ± SD.
Helix 12 in the LBD plays a major regulatory role in the activity of the ER and is involved in the interaction of the ER with E2, coregulators and inhibitors (1). Our structure model suggested a conformational change in helix 12 following the D538G substitution and predicted the mutated receptor to be constitutively active ligand-independent and bound to coactivators. The experimental data strongly support this model. Although no mutations in helix 12 have been identified in clinical samples as yet, ample laboratory data indicate the role of specific amino acids within this region. Most amino acid substitutions either do not change or decrease transcriptional activity (reviewed in ref. 3). Specifically, D538A substitution is associated with decreased activation and increased degradation of the receptor (12), and several substitutions of Tyr-537

Figure 3. Ligand-independent interaction between 538G-ERα and SRC1. A, MDA-MB-231 cells were transfected with WT-ERα, 538G-ERα, or control vector. Cells were then treated with vehicle or E2 (10 nmol/L) for 45 minutes; then cells were lysed and SRC1 was immunoprecipitated. Immunocomplexes were resolved on SDS-PAGE gel and immunoblotted with anti-ER antibodies. B, MCF-7 cells were transfected with WT-ERα, 538G-ERα, or control vector. Cells were then treated with vehicle or E2 (10 nmol/L) for 24 hours. Forty-eight hours after transfection, cells were lysed and immunoblotted with antibodies as indicated.

Figure 4. 538G-ERα enhances viability and migration of breast cancer cells. A, MCF-7 cells were seeded in 60-mm dishes and were transiently transfected with WT-ERα, 538G-ERα, or empty vector, and 24 hours later were seeded in 96-well plates. Cells then were treated with E2 (10 nmol/L) or control vehicle. Viability was assessed after 48 hours using MTT assay. * P < 0.0001 538G-ERα versus control and WT-ERα. B, MCF-7 cells were transfected as in (A) and grown to confluency in phenol-free media with 10% charcoal-treated serum. The monolayer was scraped, treated with E2 or control vehicle for 48 hours, and photographed.
alter the affinity of the ER to E2 (13). However, there are several examples for increased activation and resistance to inhibitors. For example, certain mutations of Leu-536 increased ligand-independent constitutive activity (13), and substitution of Glu-542 to alanine increased E2-dependent activity (12). Thus, the increased activity of D538G-ERα observed by us is in line with current knowledge regarding helix 12 structure and function.

D538G-ERα seems to enhance viability and migration of MCF-7 cells, even compared with E2 stimulation. It remains to be elucidated whether this phenomenon translates into more aggressive clinical course upon acquisition of the mutation. The mechanisms governing this increased activity are not known. One possibility is altered degradation. Upon E2 binding and transcriptional activity, the half-life of the ER is shortened from 5 days to 3 hours, and this may serve as a negative regulatory mode of the estrogen pathway (18). It is possible that, as D538G-ERα does not bind to the ligand, its degradation is altered and its half-life, and therefore activity, is increased.

Although no acquired mutations of ER-α were noted in the clinic, a series of articles from the laboratory of Craig Jordan reported and characterized an acquired mutation occurring in vivo. The mutation, D351Y, was discovered in MCF-7 cells grown as tumors in athymic mice chronically treated with tamoxifen (19). This is an important laboratory proof to our clinical observation. Although D351 does not reside within helix 12, upon binding of E2, D351 lies next to D538 and plays a major role in the regulation of E2-dependent and -independent activation and is also involved in mediating the agonist effects of tamoxifen (20, 21).

The presence of the mutation may represent the genetic heterogeneity of the primary tumor followed by Darwinian selection of resistance clones. This mechanism was noted in a relatively fast-growing basal-like breast cancer (22). Alternatively, de novo mutations may appear during disease progression. This was noted in a lobular breast tumor, where 32 mutations appeared during a period of 9 years (23). Regardless of the mechanism, our observation clearly indicates that genomic analyses aimed at predicting response to treatment and identifying novel potential therapeutic targets should be conducted on the most recent metastatic lesions available. Preferably, both the metastases and the primary tumor should be evaluated.

The mutation was noted only in liver metastases. In one patient, the mutation was noted in a liver metastasis and not in a pulmonary metastasis, and, in the 8 patients without the mutation, all but one sample were obtained from extrhepatic sites. This is a very preliminary observation and needs to be validated in a much larger cohort. However, it may indicate a special predilection of cancer cells with active estrogen signaling to the liver. Tropism of breast cancer cells to specific organs, especially to the lung, bone, and brain, is a well-described phenomenon (24). In accordance with our observation, a recent study noted an association between the expression of hormones receptors and liver metastases (25).

All our patients developed resistance to several forms of endocrine treatments (Table 1). Ligand-independent activity explains clinical resistance to treatment with aromatase inhibitors, which inhibit estrogen production. Resistance to tamoxifen and fulvestrant is predicted to result from a conformational change in helix 12 interfering with their binding to the receptor (Fig. 1). Studies in cell lines indicated that both tamoxifen and fulvestrant is predicted to result from a conformational change in helix 12 interfering with their binding to the receptor (Fig. 1). Studies in cell lines indicated that both tamoxifen and fulvestrant can inhibit the mutated receptor, although at high, suprapharmacologic concentrations. Thus, the concentration of tamoxifen that inhibited the mutated receptor is 1,400-fold higher than that observed in breast cancer tissue following administration of standard dose of 20 mg tamoxifen (0.07 nmol/L) and predicts complete clinical resistance to tamoxifen (26). Similarly, the effective fulvestrant concentration was higher than the plasma concentration of

Figure 5. 538G-ERα confers resistance to 4HT and fulvestrant. A, MDA-MB-231 cells were transfected with either WT-ER or 538G-ERα, together with the ERE-luciferase reporter. Cells were then treated with a vehicle control or with E2 (10 nmol/L) together with increasing doses of 4HT, as indicated, for 24 hours. Luciferase activities were analyzed and normalized to total protein concentration, and are shown relative to the control WT-ER. * P < 0.01 for WT-ERα treated with E2 alone compared to treatment with 4HT at 10 nmol/L and higher concentrations. ** P < 0.05 for 538G-ERα treated with E2 alone compared to treatment with 4HT at 100 nmol/L and higher concentrations. B, MDA-MB-231 cells were transfected as in (A), and treated with elevated fulvestrant concentrations. * P < 0.03 for WT-ERα treated with E2 alone compared to treatment with fulvestrant at 10 nmol/L and higher concentrations. ** P < 0.05 538G-ERα treated with E2 alone compared to treatment with fulvestrant at 100 nmol/L and higher concentrations. The figure shows representative results of two independent experiments, each performed in quadruplicates. Each bar represents the mean ± SD.
fulvestrant (27, 28). The observation that a high concentration of tamoxifen and fulvestrant can inhibit D538G-ERα is still encouraging and indicates that other compounds may be able to bind the receptor, prevent its activation, and restore endocrine sensitivity.

In conclusion, we identified a novel acquired mutation of ERα in metastases obtained from patients with metastatic breast cancer who developed resistance to endocrine treatment. The mutation induces a conformational change, which mimics the conformation of activated ligand-bound receptor and confers ligand-independent activity as well as resistance to endocrine treatment. This discovery may lead to the development of novel endocrine treatments for patients who develop resistance to currently available endocrine treatments.

Disclosure of Potential Conflicts of Interest

A. Dvir and L. Soussan-Gutman are employees of Oncostest-Teva Pharmaceutical Industries, the Israeli distributor of Foundation One. R. Yelensky has ownership interest (including patents) in association with Foundation Medicine. M. Brown is a consultant for and has received commercial research funding from Novartis Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

References

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