Sensitive Detection of Mono- and Polyclonal ESR1 Mutations in Primary Tumors, Metastatic Lesions, and Cell-Free DNA of Breast Cancer Patients

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Abstract

Purpose: Given the clinical relevance of ESR1 mutations as potential drivers of resistance to endocrine therapy, this study used sensitive detection methods to determine the frequency of ESR1 mutations in primary and metastatic breast cancer, and in cell-free DNA (cfDNA).

Experimental Design: Six ESR1 mutations (K303R, S463P, Y537C, Y537N, Y537S, D538G) were assessed by digital droplet PCR (ddPCR), with lower limits of detection of 0.05% to 0.16%, in primary tumors (n = 43), bone (n = 12) and brain metastases (n = 38), and cfDNA (n = 29). Correlations between ESR1 mutations in metastatic lesions and single (1 patient) or serial blood draws (4 patients) were assessed.

Results: ESR1 mutations were detected for D538G (n = 13), Y537S (n = 3), and Y537C (n = 1), and not for K303R, S463P, or Y537N. Mutation rates were 7.0% (3/43 primary tumors), 9.1% (1/11 bone metastases), 12.5% (3/24 brain metastases), and 24.1% (7/29 cfDNA). Two patients showed polyclonal disease with more than one ESR1 mutation. Mutation allele frequencies were 0.07% to 0.2% in primary tumors, 1.4% in bone metastases, 34.3% to 44.9% in brain metastases, and 0.2% to 13.7% in cfDNA. In cases with both cfDNA and metastatic samples (n = 5), mutations were detected in both (n = 3) or in cfDNA only (n = 2). Treatment was associated with changes in ESR1 mutation detection and allele frequency.

Conclusions: ESR1 mutations were detected at very low allele frequencies in some primary breast cancers, and at high allele frequency in metastases, suggesting that in some tumors rare ESR1-mutant clones are enriched by endocrine therapy. Further studies should address whether sensitive detection of ESR1 mutations in primary breast cancer and in serial blood draws may be predictive for development of resistant disease.

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See related commentary by Gu and Fuqua, p. 1034

Introduction

Estrogen receptor alpha (ERα, ESR1) is expressed in the majority of breast cancers and is a major regulator of breast cancer development and progression (1). Endocrine therapy is one of the most efficacious and least toxic treatments in ER-positive (+) breast cancers. Current strategies target ER action either by ligand deprivation [ aromatase inhibitors (AI) or ovarian function suppression] or ER blockade through selective estrogen receptor modulators (SERM) and degraders (SERD).

All these therapies may improve survival in early-stage breast cancer (2, 3). However, de novo or acquired resistance is a major clinical problem, especially in metastatic breast cancer. Multiple molecular mechanisms of resistance include downregulation of ER expression, dysregulation of ER coregulators, post-translational modifications of ER, and cross-talk with growth factor signaling pathways (4–11).

The concept that somatic base-pair missense mutations in ESR1 may confer hormone independence has been speculated for many years. However, studies of primary breast cancer have reported few or no ESR1 mutations (12–16). For example, ESR1 base-pair missense mutations are present at 0.2% (1/482) in breast cancers in The Cancer Genome Atlas (TCGA; ref. 17), and 0.38% (5/1430) in the Catalog of Somatic Mutations in Cancer. However, recent
Recent studies have identified somatic mutations in the estrogen receptor (ERs, ESR1) in endocrine-resistant metastatic breast cancer, but rarely or not at all in primary disease, suggesting that such mutations may undergo selection during endocrine therapy. Here, we report the use of highly sensitive digital droplet (ddPCR) to study the incidence of ESR1 mutation in primary breast cancer, metastatic biopsies from brain and bone, and circulating cell-free DNA (cfDNA) from breast cancer patients with advanced disease. Our results show that mutations can indeed be identified in primary disease, although at very low mutant allele fraction. In cfDNA we find that 25% of patients with advanced breast cancer harbor mutations in ESR1. Collectively, our data suggest that sensitive detection of ESR1 mutations in primary breast cancer and in serial blood draws may be predictive for development of resistant disease, a hypothesis to be tested in prospective trials.

DNA isolation, preparation, and quantification

Of note, 30 to 50 mg of frozen primary tumor tissue and 50 to 150 mg of frozen bone metastases were crushed under liquid nitrogen, and DNA was isolated using Qiagen DNAeasy Blood & Tissue Kit. Brain metastases were obtained as FFPE sections and Qiagen Allprep DNA/RNA FFPE Kit was used to isolate DNA from four to six 10 μm slides. cfDNA was isolated as previously described (29). Briefly, plasma was separated by double centrifugation within 7 days of blood collection, and DNA was isolated from 1 to 4 ml plasma using QiaAmp Circulating Nucleic Acid kit. Targeted high-fidelity preamplification (15 cycles) was performed on cfDNA and DNA isolated from FFPE brain metastases using primers listed in Supplementary Table S1. Preamplification products were purified using QiaQuick PCR purification kit and diluted before ddPCR at 1:100 and 1:20 for cfDNA and DNA, respectively. The preamplification does not affect linearity of detection of the mutant allele, as we have shown for ESR1 and PIK3CA mutations (Supplementary Fig. S1). All DNA samples were quantified by Qubit dsDNA HS/BR assay kits (Life Technologies).

Mutation detection by droplet digital PCR (ddPCR)

Primers and probes were designed and ordered through Life Technologies for S463P, Y537C/N/S, K303R, and Integrated DNA Technologies for D538G ESR1 mutations (Supplementary Table S2). Bio-Rad QX100 Droplet Digital PCR system was used. Briefly, 1 μL template from diluted preamplified products or 50 to 60 ng of nonamplified DNA was mixed with ddPCR supermix for probes (no dUTPs; Bio-Rad laboratories, Inc.) and primer/probe set. Droplets were generated using 20 μL of the reaction mixture and 70 μL of droplet generation oil. Positive and negative controls were included in each run to exclude potential contamination artifacts, and to control for proper gating of alleles. All mutation-positive samples were run in at least three replicates, assaying at least 10,000 genome equivalents. For positive controls, we utilized oligonucleotides

Materials and Methods

Sample acquisition

Samples used in this study were obtained from the University of Pittsburgh Health Sciences Tissue Bank (HSTB; Pittsburgh, PA; primary breast cancer, brain metastases), or were prospectively collected (bone metastases, blood). There were no special criteria for selection of samples for the study other than those described here. Frozen primary ER-positive breast cancers (n = 43; >60% tumor cellularity) from patients subsequently treated with endocrine therapy were obtained from HSTB. Metastatic tumor biopsies from brain (n = 38) and bone (n = 12) were collected through HSTB over the last 3 years. For collection of cfDNA (n = 29), blood was drawn (1–4 × 10 mL Streck tubes) between 01/14 and 08/14 from patients with advanced disease seen within the UPMC health system. There were a total of 122 samples, from 121 patients, since one patient (CF28) donated both cfDNA and a bone metastases sample. In addition, we had access to skin (CF4), liver (CF16), ovarian (CF23), and soft tissue neck metastases (CF14) from patients who donated blood for cfDNA isolation, thus totaling n = 126 analyzed samples. ER status was detected by IHC, using ASCO-CAP 2010 guidelines for tumors diagnosed in or after 2010 (32). All patients signed informed consent, and the studies were approved by the University of Pittsburgh IRB.
containing the mutation (463P, Y537C/N, K303R). DNA from a cell line with a D538G knock-in mutation (unpublished data), or DNA from a liver biopsy with an ESR1 mutation at Y537S confirmed by Sanger sequencing (Fig. 1). Specificity of the probes was demonstrated for Y537C/N/S and D538G mutations (Supplementary Fig. S2). No detectable cross-reactivity of mutant probes and WT probes was observed for D538G or Y537C mutation (Supplementary Fig. S3A). We did find that an increase in the presence of Y537S caused a slight downshift in the fluorescent signal for D538G (Supplementary Fig. S3B) causing a double population; however, this did not affect the calculated D538G allele frequency. The reason for the decrease in D538G fluorescence is unclear. Mutations with high allele frequencies were confirmed with Sanger sequencing using primers listed in Supplementary Table S1.

Quantitative analysis

Data were analyzed using QuantaSoft software (Bio-Rad), calculating a fractional abundance ("mutant allele frequency"). The background noise, which was higher in preamplified DNA from cfDNA and FFPE brain metastases compared with DNA from frozen tissues (primary tumors and bone metastases), was defined as the average of allele frequency plus half (for cfDNA) or full (for FFPE DNA) 95% confidence intervals (CI) of negative controls (ESR1 wild-type DNA) across all ddPCR assays. The noise was subtracted from the allele frequencies. The background noise-adjusted lower limits of detection (LLoD) of the assay were 0.05% for frozen tissues, 0.10% for cfDNA, and 0.16% for FFPE tissues (Supplementary Fig. S4). Samples were called "positive" for the ESR1 mutation if (a) the allele frequencies were >0 after subtraction of background noise, (b) >2 mutant droplets were repeatedly detected, and (c) allele frequency was > noise adjusted LLoD for at least three independent assays.

Results

ESR1 mutations in primary tumors

We screened 43 primary ER-positive tumors to detect ESR1 mutations (S463P, Y537C, Y537N, Y537S, and D538G) recently described in recurrent endocrine-resistant breast cancer. We also included the analysis of the K303R mutation, which has been previously described to be present in primary and metastatic disease, while it was not detected in other studies (33–37). Three primary tumors (PR3, PR21, PR28) were positive for D538G, with very low mutant allele frequencies between 0.07% and 0.2% (Fig. 2 and Table 1). Another sample (PR44) was positive in multiple repetitive assays, but the mutant allele frequency (0.012%) was below our LLoD. No other mutations were detected in any of the remaining primary tumors. We thus detected ESR1 mutations in 7.0% (3/43, 95% Wilson binomial CI 2%–19%) of primary ER+ breast cancers.
ESR1 mutations in bone metastases

Because decalcification of bone metastases can impact downstream analyses, we restricted our analysis of bone metastases to fresh-frozen tissue. We obtained 12 frozen bone metastases, 11 of which were from primary tumors known to be ER+. One sample (BM14) was positive for the D538G mutation, with an allele frequency of 1.4% (Fig. 2), for an overall ESR1 mutation rate in bone metastases of 8.3% (1/12; 95% CI, 0.4%–35%). Of note, the pathologist’s estimate of tumor cellularity in this sample was about 1% to 5% (Supplementary Table S3), suggesting that the allele frequency of this mutation within tumor cells in this sample is likely much higher. To confirm our ability to detect mutations across all samples, we performed an additional control by assaying for a frequent PIK3CA mutation (H1047R). Three samples (BM01, BM08, and BM11; Supplementary Table S3) tested positive for PIK3CA mutation at high allele frequencies (27.0%, 29.7%, and 37.8%), supporting suitability of our metastatic samples for mutation detection by ddPCR (Supplementary Fig. S5).

ESR1 mutations in brain metastases

We analyzed 38 brain metastases, 24 of which originated from ER+ primary tumors, and 14 from which the ER status of the primary tumors were unknown. All brain metastases with the exception of BR55 (30%–40%), BR56 (40%–60%), BR60 (40%), and BR68 (30%–50%) had at least 60% tumor cellularity. Three brain metastases (BR11, BR17, BR19) contained D538G mutations at high allele frequencies (34.3%–44.9%; Fig. 2) for an overall mutation rate of 7.9% (3/38; 95% CI, 3%–21%); all were recovered from patients with ER+ primary breast cancer giving a 12.5% frequency in disease with known ER-positivity (3/24; 95% CI, 4%–31%). The presence of the D538G mutation was confirmed by Sanger sequencing in the three brain metastases (Supplementary Fig. S6). Interestingly, sample BR17 had an additional Y537S mutation at a lower allele frequency (0.24%). Furthermore, using a dual-mutation specific probe, the mutations were found to be on separate alleles, indicative of polyclonal ESR1 mutations within a single metastatic tumor (Supplementary Fig. S7).

ESR1 mutations in cfDNA

We next interrogated ESR1 mutations in cfDNA collected from 29 patients with metastatic breast cancer, all arising from ER+ primary disease. ESR1 D538G (n = 6), Y537S (n = 2), and Y537C (n = 1) mutations were detected in a total of 7 patients, with one patient (CF4) having polyclonal ESR1-mutations consisting of Y537C, Y537S, and D538G with allele frequencies of 2.7%, 1.2%, and 5.1%, respectively (Table 1). cfDNA allele frequency was overall higher compared with primary tumors (Fig. 2). The ESR1 mutation rate in cfDNA was 24.1% (7/29; 95% CI, 12%–42%).

Table 1. The rates of ESR1 mutations in primary tumors, cfDNA, brain and bone metastases from breast cancer patients

<table>
<thead>
<tr>
<th>Samples</th>
<th>N</th>
<th>ER+ primary</th>
<th>K303R</th>
<th>S463P</th>
<th>Y537C</th>
<th>Y537N</th>
<th>Y537S</th>
<th>D538G</th>
<th>Pts with ESR1 mutation</th>
<th>Rates of ESR1 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor</td>
<td>43</td>
<td>43</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>7.0% (3/43)</td>
</tr>
<tr>
<td>Bone metastases</td>
<td>12</td>
<td>11a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>8.3% (1/12)</td>
</tr>
<tr>
<td>Brain metastases</td>
<td>38</td>
<td>24a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>7.9% (3/38)</td>
</tr>
<tr>
<td>cfDNA</td>
<td>29</td>
<td>29a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7.0% (2/29)</td>
</tr>
</tbody>
</table>

Note: aNumber with known ER+ primary tumor at time of diagnosis (ER status of primary tumor unknown for remaining samples).
bOne patient with a brain metastasis, and one with cfDNA analysis had multiple distinct ESR1 mutations within a single sample (polyclonal ESR1 mutations).
cFrequency of mutations in metastases from a known ER+ primary tumor.
Table 2. Clinical characteristics and endocrine treatment history in patients with confirmed ESR1-mutant cfDNA, brain or bone metastases

<table>
<thead>
<tr>
<th>ID</th>
<th>Specimen</th>
<th>Detected ESR1 mutations</th>
<th>Stage at Dx</th>
<th>ER Status</th>
<th>ADJ hormonal therapy duration</th>
<th>Number of therapies</th>
<th>Cumulative Exposure</th>
<th>Endocrine therapy</th>
<th>Number of therapies</th>
<th>Cumulative exposure</th>
<th>Endocrine therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF4*</td>
<td>cfDNA</td>
<td>Y537C/S, D538G</td>
<td>IIB</td>
<td>+</td>
<td>SERM</td>
<td>5</td>
<td>3</td>
<td>23</td>
<td>AI, SERM</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CF8a</td>
<td>cfDNA</td>
<td>D538G</td>
<td>IIB</td>
<td>+</td>
<td>AI</td>
<td>13</td>
<td>1</td>
<td>47</td>
<td>SERD</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>CF14a</td>
<td>cfDNA/soft tissue</td>
<td>Y537S</td>
<td>IV</td>
<td>+</td>
<td>No</td>
<td>0</td>
<td>4</td>
<td>25</td>
<td>AI, SERM, SERD</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CF16</td>
<td>cfDNA/liver</td>
<td>D538G</td>
<td>0</td>
<td>+</td>
<td>No</td>
<td>0</td>
<td>4</td>
<td>35</td>
<td>AI, SERM, SERD</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>CF23</td>
<td>cfDNA</td>
<td>D538G</td>
<td>IV</td>
<td>+</td>
<td>No</td>
<td>0</td>
<td>3</td>
<td>42</td>
<td>AI, SERD</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>CF27a</td>
<td>cfDNA</td>
<td>D538G</td>
<td>IV</td>
<td>+</td>
<td>No</td>
<td>0</td>
<td>7</td>
<td>57</td>
<td>AI, SERM, SERD</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>BR1</td>
<td>Brain</td>
<td>D538G</td>
<td>0</td>
<td>+</td>
<td>No</td>
<td>0</td>
<td>4</td>
<td>47</td>
<td>AI, SERM, SERD</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>BM14/CF28</td>
<td>cfDNA/bone</td>
<td>D538G</td>
<td>III</td>
<td>+</td>
<td>AI</td>
<td>72</td>
<td>2</td>
<td>9</td>
<td>AI, SERD</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>BR19</td>
<td>Brain</td>
<td>D538G</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>BR17</td>
<td>Brain</td>
<td>Y537S, D538G</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td>NK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ADJ, adjuvant; AI, aromatase inhibitor; Dx, diagnosis; NK, not known; SERD, selective estrogen receptor downregulator; SERM, selective estrogen receptor modulator. *Patient is deceased.

in cfDNA. Typical of patients with ER$^+$ metastatic breast cancer, most had an extensive history of endocrine therapy as measured by both number of agents and months of exposure. There were not sufficient number of samples to formally analyze a predicted association between ESR1 mutations and shorter survival.

Analysis of ESR1 mutations in serial blood samples, and matched metastatic tumors

Serial blood draws from 4 patients were available for longitudinal examination of ESR1 mutation status. Patient CF4 (Fig. 3A) was originally diagnosed with ER$^+$ lymph-node positive disease, underwent mastectomy, and was then treated with SERMs. Over the next year, she developed metastases to brain, liver, bone, and skin. A metastatic skin lesion biopsy was negative for ESR1 mutation. A blood draw 6 months later showed three ESR1 mutations with different allele frequencies (Y537C 2.7%, Y537S 1.2%, D538G 5.1%). The patient received an aromatase inhibitor, everolimus, and chemotherapy for 6 months. Subsequent blood draw (6 months after the first one) revealed an enrichment of Y537C and D538G mutations, but a loss of the Y537S-mutant clone (Y537C - 7.4%, Y537S < LLoD, D538G - 10.1%). The increase in the allele frequencies of D538G and Y537C co-occurred with an increase in the tumor marker CA 27-29.

For patient CF16, DNA from five serial blood draws and from a biopsy of a liver metastasis was analyzed (Fig. 2B). The patient originally developed ER$^+$ chest wall metastases 12 years after excision of DCIS. She received serial endocrine therapy including tamoxifen, fulvestrant, and multiple AIs, followed by mTOR inhibitor and chemotherapy, but metastases progressed to other sites, including liver and bone. The ESR1 D538G mutation was detected in both the liver metastasis (23.0%) and the first blood draw (1.0%). The allele frequency was similar in the second blood draw (0.9%), peaked around the time of the third draw (13.7%), decreased in the fourth blood draw (4.9%), and was below LLoD in the fifth draw taken after approximately 6 months of chemotherapy (0.2% before noise subtraction, which did not pass the cut-off for "positive" mutation calling). The decreased frequency of the mutant allele corresponded to lower CA 27-29 levels after chemotherapy.

Two additional patients (CF23, CF28) had two blood draws each (Supplementary Fig. S8). Patient CF23 presented with Stage IV disease, with multiple bone lesions, and an ovarian metastasis that was negative for ESR1 mutation. Blood was drawn at two time points throughout disease progression, as indicated in Supplementary Fig. S8A, which was approximately 1 month after surgical removal of the ovarian metastasis. D538G mutation was detected at low allele frequency (0.2%) in the first draw, and was below LLoD in the second draw. Patient CF28 developed lung, bone, and brain metastases 3 years after completion of 5 years of AI treatment for an ER$^+$ breast tumor (Supplementary Fig. S8B). She was treated with AI, and fulvestrant, and cfDNA from first blood draw was negative for ESR1 mutations. The disease progressed, and a subsequent bone biopsy revealed an ESR1 D538G mutation (1.4% allele frequency; BM14, described above), and cfDNA showed the D538G mutation at 7.8% allele frequency. The increase in allele frequency of D538G co-occurred with an increase in CA 27-29 tumor marker. Finally, in one additional patient (CF14) with a single blood draw, the Y537S mutation was detected in both a posterior neck soft tissue nodule (40.5% allele frequency), and in cfDNA, although at lower frequency (0.8%).

Thus, in summary, mutations were either detected in both metastatic biopsy and cfDNA (n = 3) or in cfDNA only (n = 2), suggesting cfDNA as a source for disease phenotyping (e.g., detecting types of mutations), and potentially monitoring burden. This is supported by the observation that changes in ESR1 mutation frequency correlated with changes in CF27-29 levels.

Discussion

ESR1 mutations are present at very low allele frequency in primary ER-positive breast cancer

Previous studies have shown low or undetectable rates of ESR1 mutation in primary breast cancer using Sanger sequencing or massively parallel sequencing (MPS). This is the first study to examine ESR1 mutations (S463P, Y537C, Y537N, Y537S, and D538G, K303R) in primary breast cancer using ddPCR. We found...
that 7.0% (3/43) of primary breast cancers have an ESR1 D538G mutation, but the allele frequency is very low (0.07%–0.2%). A recent NGS study of primary tumors from BOLERO trial identified ESR1 mutation in 6 of 183 tumors (3.3%; 22). TCGA did not detect ESR1 D538G mutation (or K303R, S463P, Y537C, Y537N, Y537S) in 482 primary breast cancers, and COSMIC contains only one ESR1 D538G mutation from 1,430 primary breast cancers. The very low allele frequency suggests that in some primary tumors, ESR1 mutations preexist as rare clones, which are then selected for during metastatic progression. This is consistent with a previous study from a single patient, which used deep-targeted MPS and identified an ESR1 mutation (E380Q) at 2% allele frequency as measured by ddPCR. The most frequently identified ESR1 mutations in these speciﬁc metastatic sites and the only one to use ddPCR. The most frequently identiﬁed ESR1 mutation was D538G, which is consistent with prior studies that detected a total of fourteen D538G mutations, eleven Y537S mutations, four Y537N mutations, three Y537C mutations, two S463P mutations, and eight other ESR1 mutations in a total of 329 samples (18–22). The slightly increased rate of D538G mutations compared with other mutations may be a result of the small sample size in our study. We did not detect the K303R mutation in any of our 126 analyzed samples. The prevalence of K303R has been controversial with one group reporting high frequencies of up to 34% (34) and 50% (38) in premalignant and invasive breast cancer respectively, while others have identiﬁed it at low frequency (33, 39), or not at all (18–22, 35–37, 40). The sensitivity of our detection methods suggests that the occurrence of the K303R mutation is likely to be rare.

We detected very high allele frequency (34.3%–44.9%) in brain metastases, indicating that the ESR1-mutant clones are likely dominant clones, and suggesting that the ESR1 mutation is a driver event in metastatic progression to this site. Only one bone metastasis had an ESR1 mutation of relatively low allele frequency (1.4%); however, this low frequency is likely due to the very low tumor cellularity in this sample (1%–5%). In the future, it might be of interest to test whether different ESR1 mutations preferentially seed at different metastatic sites.

**ESR1 exhibits polyclonal mutations**

Previous studies have shown convergent evolution of polyclonal mutations in cancer, with different mutations in the same
gene ultimately targeting the same phenotype (41). We observed cases with multiple ESR1 mutations in the same tumor, and demonstrated that mutations (Y537S and D538G) were on different alleles, indicating polyclonal disease. Patient CF4 is unique in that cfDNA contained three different ESR1 mutations. It is possible that the cfDNA integrates ESR1 mutations from distinct populations of cells, potentially arising from different metastases. The presence of three different mutations in the ligand-binding domain of ESR1 highlights the substantial selection pressure for these types of mutations during endocrine therapy. Interestingly, longitudinal analysis of cfDNA in this patient indicated increased mutant allele frequency of two clones, and loss of the third clone, possibly reflecting differential response of individual ESR1 mutations to treatments. There is some prior evidence for different biologies of the different mutants. Toy and colleagues show that ligand-independent activity of Y537S is stronger than that of D538G, and weak for S463P (22). It will be important to investigate whether this polyclonality is important in treatment response and tumor progression, for example, if different clones support each other, or if this simply represents a snapshot of a high rate of genomic instability.

Longitudinal monitoring of ESR1 mutations in cfDNA
We detected ESR1 mutations at high mutant allele frequency in cfDNA from patients with advanced breast cancer. The ease of obtaining cfDNA and the high sensitivity suggest that this may be a valuable tool for detecting ESR1 mutations in patients with advanced breast cancer. However, larger studies directly comparing ESR1 mutation in paired cfDNA and metastatic tumor biopsies are required to confirm this possibility. In addition, cfDNA analysis potentially affords an invaluable approach for longitudinal measurement of mutations that is simply not possible with solid biopsies. This is shown, for example, in patient CF4 where an initial skin biopsy was negative for ESR1 mutations. This is shown, for example, in patient CF4 where an initial skin biopsy was negative for ESR1 mutations. This is shown, for example, in patient CF4 where an initial skin biopsy was negative for ESR1 mutations.

Disclosure of Potential Conflicts of Interest
R.J. Hartmaier reports receiving speakers bureau honoraria from BioRad. S. Puhalla reports receiving other commercial research support from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Disclaimer
The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies or the University of Pittsburgh.

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Study supervision: K. Weiss, A.M. Stern, S. Oesterreich

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