**ESR1 Mutations in Circulating Plasma Tumor DNA from Metastatic Breast Cancer Patients**

David Chu¹, Costanza Paoletti², Christina Gersch², Dustin A. VanDenBerg¹, Daniel J. Zabransky¹, Rory L. Cochran¹, Hong Yuen Wong², Patricia Valda Toro¹, Justin Cidado¹, Sarah Crossmann¹, Bracha Erlanger¹, Karen Cravero¹, Kelly Kyker-Snowman¹, Berry Button¹, Heather A. Parsons¹, W. Brian Dalton¹, Riaz Gillani¹, Arielle Medford¹, Kimberly Aung², Nahomi Tokudome², Arul M. Chinnaiyan², Anne Schott², Dan Robinson², Karen S. Jacks³, Josh Lauring¹, Paula J. Hurley¹, Daniel F. Hayes², James M. Rae², and Ben Ho Park¹,⁴

**Abstract**

**Purpose:** Mutations in the estrogen receptor (ER) gene, ESR1, have been identified in breast cancer metastases after progression on endocrine therapies. Because of limitations of metastatic biopsies, the reported frequency of ESR1 mutations may be underestimated. Here, we show a high frequency of ESR1 mutations using circulating plasma tumor DNA (ptDNA) from patients with metastatic breast cancer.

**Experimental Design:** We retrospectively obtained plasma samples from eight patients with known ESR1 mutations and three patients with wild-type ESR1 identified by next-generation sequencing (NGS) of biopsied metastatic tissues. Three common ESR1 mutations were queried for using droplet digital PCR (ddPCR). In a prospective cohort, metastatic tissue and plasma were collected contemporaneously from eight ER-positive and four ER-negative patients. Tissue biopsies were sequenced by NGS, and ptDNA ESR1 mutations were analyzed by ddPCR.

**Results:** In the retrospective cohort, all corresponding mutations were detected in ptDNA, with two patients harboring additional ESR1 mutations not present in their metastatic tissues. In the prospective cohort, three ER-positive patients did not have adequate tissue for NGS, and no ESR1 mutations were identified in tissue biopsies from the other nine patients. In contrast, ddPCR detected seven ptDNA ESR1 mutations in 6 of 12 patients (50%).

**Conclusions:** We show that ESR1 mutations can occur at a high frequency and suggest that blood can be used to identify additional mutations not found by sequencing of a single metastatic lesion. *Clin Cancer Res;* 1–7. ©2015 AACR.

**Introduction**

Estrogen receptor (ER)-α is a part of the nuclear hormone receptor family and is expressed in about 70% of breast cancers (1). Drugs that target ER and estrogen production have become effective standard-of-care therapies (2). Notably, selective estrogen receptor modulators (SERM), selective estrogen receptor downregulators (SERD), and aromatase inhibitors (AI) have significantly improved overall survival of patients with ER-positive breast cancer (3). Nevertheless, *de novo* and acquired resistance may arise after prolonged exposure to these therapies (4). Recently, next-generation sequencing (NGS) studies of patients with metastatic ER-positive breast cancer have revealed genetic alterations that may account for acquired resistance to endocrine therapy (5–9). These studies collectively report mutations in the ligand-binding domain (LBD) of ESR1 in approximately 20% of these patients, and presumably these mutations act as a driver of endocrine therapy resistance. Interestingly, these mutations were predicted in mutagenesis models and identified in patient xenograft studies reported almost two decades ago (10, 11). Molecular modeling and preclinical studies characterizing ESR1 LBD mutations reveal a conformational change that leads to constitutive activation of ER signaling in the absence of ligand (6–8). However, these studies also suggest that cells with ESR1 LBD mutations may still be sensitive to SERM and SERD therapy, albeit at higher doses compared with cells with wild-type ESR1 (7, 8). The identification of ESR1 mutations that are responsible for endocrine therapy resistance in ER-positive breast cancers opens the door for developing new diagnostic tools and novel targeted therapies. However, given the problem of tumor heterogeneity,
the true frequency of ESR1 mutations may be underestimated, as mutational profiles can vary between different sites of metastatic disease (12). Most studies heretofore have used NGS of a single metastatic site, and indeed, one study demonstrated an ESR1 mutation in a liver metastatic biopsy but not a lung metastasis obtained from the same patient (6). Furthermore, in many cases, fresh biopsies of metastatic cancer cannot be safely obtained and/or archival tissues are inadequate or unavailable. Finally, these mutations appear to evolve during endocrine treatment, and therefore a noninvasive method of monitoring patients might provide an opportunity to alter therapy as these mutations emerge. Thus, there is a need to develop noninvasive methods to quickly assess mutational profiles across multiple metastases from an individual patient.

Recently, we and others have examined the use of circulating cell-free plasma tumor DNA (ptDNA) as a biomarker for cancer detection (13–20). It is known that DNA molecules from both normal and cancer cells are shed or released into the circulation (21, 22). Because DNA from cancer cells harbor somatic mutations and rearrangements, these can serve as specific genetic biomarkers for the presence of cancer. Furthermore, the quantity of ptDNA directly correlates with tumor burden and response to therapies (23). In addition, several groups have demonstrated the ability to detect the presence of acquired drug resistance mutations in ptDNA (24, 25), which opens the possibility for earlier therapeutic intervention. More recently, our group has shown that a next-generation digital PCR platform, termed droplet digital PCR (ddPCR) has exquisite sensitivity and specificity for detecting cancer mutations in patients with early-stage breast cancer (19). We hypothesized that ddPCR could be a more sensitive platform for ESR1 mutation detection in patients with metastatic breast cancer and may show a more accurate frequency of these mutations in ER-positive disease. To test this hypothesis, we performed ddPCR for ESR1 mutations on cell-free plasma samples from patients with metastatic breast cancer and compared ESR1 mutations in ptDNA with NGS of metastatic tumor tissue from the same patients.

Translational Relevance
ESR1 mutations can arise in estrogen receptor (ER)–positive breast cancer metastases after progression on endocrine therapies. However, because of tumor heterogeneity and difficulty in obtaining metastatic biopsies, a “liquid biopsy” using circulating plasma tumor DNA (ptDNA) would facilitate assessment of ESR1 mutations. We developed a blood-based assay to detect ESR1 mutations using droplet digital PCR (ddPCR) and compared the results with next-generation sequencing (NGS) of metastatic tissue biopsies in patients with breast cancer. In a retrospective cohort (n = 11), we detected all mutations in blood that were present in tissues by NGS and discovered two additional ESR1 mutations in ptDNA samples. In a prospective cohort (n = 12), we identified seven ESR1 mutations in blood and no mutations were detected in metastatic biopsies. These results demonstrate a higher frequency of ESR1 mutations in ptDNA than in corresponding metastatic biopsies and suggest that ddPCR of ptDNA may be preferred for ESR1 mutation detection.

Materials and Methods
Patient and sample collection
We conducted this clinical study at the University of Michigan Comprehensive Cancer Center (UMCCC; Ann Arbor, MI) and the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center (JHKS; Baltimore, MD). Men and women with metastatic (stage IV) breast cancer were eligible. All patients signed informed consent. For the UMCCC cohort, patients were recruited from patients with breast cancer undergoing a research tumor biopsy of metastatic disease for whole-exome sequencing through UMCCC’s MiONCOSEQ program (7, 26). In particular, these patients were recruited in a companion trial to MiONCOSEQ, designated MiCTC-ONCOSEQ approved by the University of Michigan Health System IRB. Under this protocol, any patient with metastatic breast cancer previously enrolled or enrolling in the parent MiONCOSEQ protocol was asked to provide blood samples for ptDNA collected in BCT DNA tubes (Streck) and circulating tumor cell (CTC) analyses (data not reported in this publication). For the JHKS cohort, patients were consented and enrolled in an ongoing longitudinal tissue and blood repository protocol, allowing for research use of human tissues and bodily fluids from patients with breast disease. An IRB subprotocol approved for genomic analyses of tumor tissues and blood from patients with breast cancer of any stage was used to obtain metastatic tumor biopsies and subsequent blood samples from ER-positive metastatic patients. Metastatic tumor samples obtained as formalin-fixed, paraffin-embedded (FFPE) blocks and slides were sent for NGS DNA analysis using a commercial source (Foundation Medicine). In this cohort, blood samples of 30 mL were collected in EDTA tubes or BCT DNA tubes after patients with ESR1 mutations were identified. Prospective enrollment is also allowed for this protocol.

Isolation and quantification of ptDNA for ddPCR
Blood samples and plasma DNA preparation were performed as previously described (19). Briefly, plasma was obtained by a double-spin centrifugation protocol of whole blood to remove cellular contaminants and DNA extracted using the Qiagen Circulating Nucleic Acid Kit (Qiagen) per the manufacturer’s protocol. Blood was centrifuged within 1 hour if collected in EDTA tubes and within 7 days if collected in DNA BCT tubes (Streck). The Bio-Rad QX200 platform was then used for ddPCR per the manufacturer’s protocol, with results reported as a percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild-type) DNA alleles as previously described (19). Further details are provided in Supplementary Methods.

Statistical analysis
To quantify the percentage of ptDNA containing mutant ESR1 in plasma samples, a fractional abundance calculation using the QuantaSoft program (Bio-Rad Technologies) was used, using the total number of droplets (with and without DNA) to calculate the number of DNA molecules as copies/μL and then dividing the number of mutant DNA molecules by the number of total DNA molecules (mutant plus wild-type) multiplied by 100 to yield a percentage of mutant DNA molecules in a sample taking into account a Poisson distribution of occupied to unoccupied droplets. For cohort 2, Fisher exact two-tailed test (GraphPad) was used to calculate differences in ESR1 mutation status (mutant vs.
Results

We enrolled a total of 23 patients in two separate cohorts (Fig. 1) from UMCCC and JHSKCCC. Systemic endocrine therapies are shown (Table 1), although many patients also received prior chemotherapies. To determine whether we could identify circulating ESR1 mutations in patients with known tissue ESR1 status, we initially performed a retrospective analysis by obtaining plasma samples from 11 patients who had previously undergone NGS of a metastatic lesion (Table 2). Plasma DNA was obtained from these patients less than 1 year after their tissue biopsy. To determine whether we could identify ESR1 mutations in metastatic disease (9). As demonstrated in Supplementary Fig. S1, each probe was specific for its respective mutation using Y537S, Y537N, and D538G mutant and wild-type templates. As shown in Table 2, ddPCR successfully detected all mutations in ptDNA that were detected in the metastatic biopsy, confirming the ability to detect mutations present within the tumor sample. The majority of patients had significant tumor burden with multiple metastatic sites of disease (Supplementary Table S1), although patient 5 had no evidence of disease after removal of her metastatic lesion. Indeed, although she did have a circulating ESR1 mutation (D538G), it was detected at a relatively low fractional abundance (0.03%) in her plasma.

In addition to harboring the known tissue mutation (Y537S in her circulation), patient 1 also had a low fractional abundance (0.01%) of a second circulating mutation, D538G, which was not detected in the metastatic tissue. It should be noted, however, that her blood was drawn 186 days after biopsy, and thus a new subclonal population within the same metastatic site could have been present at the time of blood draw. Similarly, patient 9 was wild-type for ESR1 in the metastatic lesion but showed a D538G mutation at a relatively low fractional abundance in a plasma sample obtained 54 days after biopsy. These results suggest that ddPCR of ptDNA can reliably detect ESR1 mutations first identified in metastatic tissues and may also detect subclonal populations in the metastatic biopsy below the limit of detection by NGS or mutations from other sites of disease.

The presence of two additional mutations in patients 1 and 9 may have been due to clonal evolution in the interim between tissue biopsy and blood draw for ptDNA analysis. To address this possibility, we prospectively enrolled 8 additional ER-positive patients (patients 12–19) to simultaneously collect metastatic tissue biopsies and blood for NGS and ddPCR analysis, respectively. As controls, we also obtained metastatic tissue and blood samples from 4 ER-negative patients (patients 20–23). All patients were enrolled at UMCCC except patient 19 who was enrolled at JHSKCCC. As shown in Table 3, sufficient tissue could not be obtained for patients 12 and 19, whereas patient 14 did not have adequate sample for NGS analysis. These patients highlight the fact that metastatic biopsies are not always obtainable and that the amount of tissue can preclude genomic analysis.

After plasma DNA extraction, ddPCR analysis was performed in a blinded fashion. As seen in Table 3, all patients had blood drawn at the time of tissue biopsy, except 2 patients (patients 20 and 21) who had blood drawn 5 and 3 days after biopsy, respectively, for logistical reasons. Of the 5 ER-positive patients for whom tissue NGS results could be obtained, no ESR1 mutations were identified in their metastatic biopsies. However, ESR1 mutations were detected in the ptDNA samples from 3 of these patients (patients 13, 16, and 18), all of whom had their blood drawn the same day as biopsy. Of note, patient 16 was a known germline BRCA2 mutation carrier and may have had a primary peritoneal (ovarian) carcinoma concurrent with her liver metastases, thus obfuscating the origin of the liver lesion. As expected, the ER-negative patients (patients 20–23) did not have detectable ESR1 mutations in their
separate alleles. To prove this, we developed a dual mutation-

clonal populations. Analysis of ptDNA from patients 1 and

Interestingly, patient 14, who was ER-positive, had a high
cant between tissue and blood using two-tailed Fisher exact

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at study entry (median = 58), y</th>
<th>Site of tissue biopsy</th>
<th>Primary ER/PR/HER2</th>
<th>Metastatic ER/PR/HER2</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>Peritoneal fluid</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen × 5 y (adjuvant), letrozole (metastatic), tamoxifen (metastatic), fulvestrant, exemestane + everolimus</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>Liver</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen × 4 y (adjuvant), anastrozole (metastatic), fulvestrant, estrace, exemestane</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>Liver</td>
<td>+/+/-</td>
<td>+/+/+</td>
<td>Tamoxifen × 5 y (adjuvant), letrozole (metastatic)</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>Liver</td>
<td>NA</td>
<td>+/+/-</td>
<td>Tamoxifen and zolendronic acid × 4 y, anastrozole × 6 wk, fulvestrant × 1 y, exemestane × 2 mo</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>Liver</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen × 2 y (adjuvant), letrozole (+ sorafenib on trial) × 4 mo, anastrozole × 1 y, fulvestrant × 4 mo</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>Brain</td>
<td>+/+/NA</td>
<td>+/+/-</td>
<td>Tamoxifen × 1 y, letrozole × 1 y, fulvestrant × 6 mo</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>Liver</td>
<td>+/+/NA</td>
<td>+/+/-</td>
<td>Tamoxifen (adjuvant), letrozole, exemestane + everolimus, fulvestrant (many years)</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>Liver</td>
<td>+/+/NA</td>
<td>+/+/-</td>
<td>Tamoxifen × 5 y (adjuvant), letrozole × 5 y (adjuvant), anastrozole × 1.5 y, fulvestrant × 8 mo</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>Sternal mass</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen (adjuvant), anastrozole (adjuvant), fulvestrant + anastrozole (metastatic)</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>Skin and subcutaneous tissue</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen × 5 y (adjuvant), exemestane, fulvestrant (metastatic)</td>
</tr>
<tr>
<td>11</td>
<td>58</td>
<td>Bone</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen × 2 y (adjuvant), anastrozole (adjuvant), fulvestrant (metastatic)</td>
</tr>
<tr>
<td>Cohort 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>61</td>
<td>Unable to get tissue for analysis</td>
<td>+/NA/NA</td>
<td>+/+/-</td>
<td>Tamoxifen × 5.5 y (adjuvant), letrozole × 1.5 y (adjuvant), anastrozole (adjuvant), fulvestrant + anastrozole (metastatic)</td>
</tr>
<tr>
<td>13</td>
<td>63</td>
<td>Periaortic lymph node</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Tamoxifen × 3 y (adjuvant), exemestane × 5 y (adjuvant), anastrozole (metastatic), fulvestrant, exemestane</td>
</tr>
<tr>
<td>14</td>
<td>48</td>
<td>Pleural fluid</td>
<td>+/+/-</td>
<td>NA</td>
<td>Tamoxifen (metastatic), fulvestrant + leuprolide, fulvestrant + anastrozole + leuprolide</td>
</tr>
<tr>
<td>15</td>
<td>56</td>
<td>Right axillary lymph node</td>
<td>+/+/-</td>
<td>+/NA/-</td>
<td>Letrozole + goserelin (neoadjuvant), letrozole + goserelin (adjuvant), fulvestrant (metastatic), fulvestrant + letrozole</td>
</tr>
<tr>
<td>16</td>
<td>77</td>
<td>Liver</td>
<td>+/+/+</td>
<td>+/+/-</td>
<td>Tamoxifen × 5 y, exemestane (adjuvant), anastrozole (adjuvant)</td>
</tr>
<tr>
<td>17</td>
<td>63</td>
<td>Axillary lymph node</td>
<td>+/-</td>
<td>NA</td>
<td>Anastrozole (metastatic), fulvestrant, tamoxifen, exemestane + everolimus</td>
</tr>
<tr>
<td>18</td>
<td>65</td>
<td>Subcutaneous chest wall nodule</td>
<td>+/+/-</td>
<td>+/+/-</td>
<td>Anastrozole (adjuvant), tamoxifen (adjuvant), fulvestrant (metastatic)</td>
</tr>
<tr>
<td>19</td>
<td>65</td>
<td>Unable to get tissue for analysis</td>
<td>+/+-</td>
<td>NA</td>
<td>Letrozole × 5 y (metastatic), fulvestrant</td>
</tr>
<tr>
<td>20</td>
<td>63</td>
<td>Skin</td>
<td>-/+/-</td>
<td>-/+/-</td>
<td>None</td>
</tr>
<tr>
<td>21</td>
<td>41</td>
<td>Lung, right lower lobe</td>
<td>-/+/-</td>
<td>-/+/-</td>
<td>None</td>
</tr>
<tr>
<td>22</td>
<td>49</td>
<td>Right anterior chest wall</td>
<td>-/+/-</td>
<td>-/+/-</td>
<td>None</td>
</tr>
<tr>
<td>23</td>
<td>57</td>
<td>Liver</td>
<td>-/+/-</td>
<td>-/+/-</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

ptDNA. The difference in mutational status was statistically sign-
nificant between tissue and blood using two-tailed Fisher exact
test (P < 0.0186).

Interestingly, patient 14, who was ER-positive, had a high
fractional abundance of two distinct circulating ESR1 mutations
(Y537S, 5.02%; D538G, 2.62%). Her only metastatic site ame-
nable to biopsy was a pleural effusion, which was inadequate for
NGS. The ptDNA from this patient collected concurrently at the
time of biopsy contained two distinct mutations at differing allelic
frequencies, suggestive of two separate clonal populations. This
was similar to patient 1 and suggestive that the mutations were on
separate alleles. To prove this, we developed a dual mutation-
specific probe and positive control template. As shown in Sup-
plementary Fig S2, this probe has specificity for a synthetic allele
harboring both mutations. Analysis of ptDNA from patients 1 and
14 using this probe showed no positive signals, demonstrating
that the two ESR1 mutations are on separate alleles, further
supporting that these ESR1 mutations are derived from different
clonal populations.

An additional noteworthy case is patient 19, who presented
at the time of diagnosis with widespread, bone-only ER-pos-
itive metastatic disease. She initiated treatment with the aro-
matase inhibitor letrozole, and after 1 year of therapy, restaging
scans showed disease stabilization of her bony metastasis and
complete resolution of her breast tumor. She elected to have bilateral mastectomies, which revealed that the affected breast and the contralateral breast had no evidence of disease. She remained on letrozole for 5 years with stable disease. She enrolled in our study while still in remission, although restaging scans continued to demonstrate only prior bony lesions, which were not amenable to biopsy. Nonetheless, her plasma demonstrated the presence of the Y537N mutation. Because of her unusual presentation, this is the only patient in our cohort that had developed an ESR1 mutation after exposure to a single endocrine therapy, letrozole. Subsequently, she had an asymptomatic elevation in her tumor markers and her therapy was changed to fulvestrant. Clinically, she remains without evidence of progression and has had stabilization of tumor markers. Although other studies have suggested that aromatase inhibitors may be the class of endocrine therapies that selects for LBD ESR1 mutations (27), most studies have enrolled patients who have received multiple lines of endocrine therapy in both the adjuvant and metastatic settings, which precludes any definitive conclusions. This patient demonstrates that an ESR1 mutation can indeed occur after prolonged exposure to an aromatase inhibitor without other endocrine or systemic therapies and that ESR1 mutations do not necessarily preclude response to a subsequent fulvestrant.

### Discussion

There are several important conclusions with potential therapeutic implications derived from our study. First, we have demonstrated that ESR1 mutations can be readily detected using ddPCR on plasma from patients with metastatic ER-positive disease after progression on endocrine therapies. Given challenges that can arise in obtaining a metastatic biopsy as encountered in this study, the use of ptDNA as a “liquid biopsy” holds great promise for future molecular analysis of human cancers. Moreover, monitoring for emergence of mutated clones by repeat sampling can be more easily performed with a simple blood test than with multiple tissue biopsies. Second, we demonstrate that blood can be a more sensitive source for detecting ESR1 mutations. In our study, 2 patients harbored a distinct, second ESR1 mutation not present in the corresponding metastatic biopsies. Perhaps more importantly, 1 patient in cohort 1 and 3 patients in cohort 2 had wild-type ESR1 in their metastatic biopsies but had ESR1 mutations detected in their corresponding ptDNA sample. These results support the increasingly recognized problem of tumor heterogeneity and are in agreement with a prior report demonstrating differences in ESR1 mutation status between two metastatic sites within the same patient (6). Third, our results support the previously proposed hypothesis that ESR1 LBD mutations may be selected for after progression on aromatase inhibitors.

### Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days between tissue biopsy and blood draw</th>
<th>Sequencing FFPE tumor tissue</th>
<th>ddPCR plasma for ESR1 Y537S</th>
<th>ddPCR plasma for ESR1 Y537N</th>
<th>ddPCR plasma for ESR1 D538G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>186</td>
<td>ESR1 Y537S</td>
<td>Y537S (0.87%)</td>
<td>Wild-type</td>
<td>D538G (0.01%)</td>
</tr>
<tr>
<td>2</td>
<td>344</td>
<td>ESR1 Y537S</td>
<td>Y537S (1.69%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>3</td>
<td>275</td>
<td>ESR1 D538G</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>D538G (1.55%)</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>ESR1 Y537S</td>
<td>Y537S (0.63%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>ESR1 D538G</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>D538G (0.03%)</td>
</tr>
<tr>
<td>6</td>
<td>165</td>
<td>ESR1 D538G</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>D538G (4.25%)</td>
</tr>
<tr>
<td>7</td>
<td>88</td>
<td>ESR1 D538G</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>D538G (0.01%)</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>ESR1 Y537N</td>
<td>Wild-type</td>
<td>Y537N (0.68%)</td>
<td>Wild-type</td>
</tr>
<tr>
<td>9</td>
<td>54</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>D538G (0.01%)</td>
</tr>
<tr>
<td>10</td>
<td>145</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>11</td>
<td>270</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>

NOTE: Cohort 1: ESR1 mutations in metastatic tissues are present in ptDNA from blood within 1 year of biopsy and additionally, blood samples were analyzed by ddPCR for ESR1 Y537S, Y537N, and D538G mutations. Percentage reflects the fractional abundance of mutant ESR1 (Y537S, Y537N or D538G) to total ESR1 DNA.

### Table 3

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days between tissue biopsy and blood draw</th>
<th>Sequencing FFPE tumor tissue</th>
<th>ddPCR plasma for ESR1 Y537S</th>
<th>ddPCR plasma for ESR1 Y537N</th>
<th>ddPCR plasma for ESR1 D538G</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>n/a</td>
<td>Y537S (0.47%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>Wild-type</td>
<td>Y537S (5.02%)</td>
<td>Wild-type</td>
<td>D538G (0.01%)</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>n/a</td>
<td>Y537S (0.02%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>Wild-type</td>
<td>Y537S (0.02%)</td>
<td>Wild-type</td>
<td>D538G (2.62%)</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>Wild-type</td>
<td>Y537S (0.02%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>Wild-type</td>
<td>Y537S (0.02%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>Wild-type</td>
<td>Y537S (0.02%)</td>
<td>Wild-type</td>
<td>D538G (0.01%)</td>
</tr>
<tr>
<td>19</td>
<td>—</td>
<td>n/a</td>
<td>Y537S (0.06%)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
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NOTE: Cohort 2: ESR1 mutations are present in ptDNA in patients with wild-type ESR1 metastatic biopsies when obtained contemporaneously and additionally, blood samples were analyzed by ddPCR for ESR1 Y537S, Y537N, and D538G mutations. Percentage reflects the fractional abundance of mutant ESR1 (Y537S, Y537N or D538G) to total ESR1 DNA. Abbreviation: n/a, not available.
inhibitors (7). This was particularly striking in patient 19, who was positive for an ESR1 mutation and had received only prolonged exposure to letrozole. Fourth, our study shows that ddPCR of ptDNA is capable of detecting ESR1 mutations even in patients who have no radiographic evidence of disease. Although the clinical validity and utility of this observation remain to be proven, we suggest that detecting drug-resistant mutations may afford the opportunity to change therapies earlier or enroll in trials of novel targeted therapies, which may lead to improved outcomes for patients. Finally, the frequency of circulating ESR1 mutations in our study is notably higher than prior reports using a single metastatic biopsy. The majority of studies thus far have detected ESR1 mutations only in patients with metastatic disease after progression on endocrine therapies, although one study did find a low incidence (3%) in primary tumors (8). The largest study to date of ESR1 mutations in metastatic tissue biopsies suggests an overall frequency of 12%, with a frequency of 20% in a subgroup analysis of patients who received an average of 7 lines of therapy (9). However, we found additional mutations not detected by sequencing of metastatic lesions. In cohort 1, two additional mutations were discovered: patient 1 who had an additional ESR1 mutation found in ptDNA compared with her metastatic biopsy and patient 9 who was wild-type for ESR1 on her metastatic tissue sample. In addition, in cohort 2, we detected seven ESR1 mutations in 6 of the 8 ER-positive patients not detected in metastatic biopsies, although 3 of these patients did not have adequate tissue for NGS. These results highlight the potential impact of using blood as a more sensitive and accessible source for mutation detection.

The higher frequency of ESR1 mutations in blood compared with biopsied tissues could be due to several nonoverlapping reasons. As mentioned, tumor heterogeneity can lead to the detection of mutations in ptDNA that are present in other nonbiopsied metastatic sites. It is also conceivable that sampling error of biopsies may miss subclonal populations in a given metastatic lesion, and/or certain clonal populations may have a propensity for releasing ptDNA versus other clonal variants. For example, it is possible that ptDNA shed from CTCs is more abundant than ptDNA derived from other metastatic sites. Further studies are needed to clarify the origins and kinetics of ptDNA as related to sites of metastases, and any underlying biology that may favor the enrichment of clonal populations that shed versus lower amounts of ptDNA into the circulation.

There are limitations of our study, most notably the small sample size, which prevents our assessing the true prevalence of ESR1 mutations in plasma from patients with ER-positive breast cancer. Furthermore, we only queried for the three most common ESR1 LBD mutations, and it is likely ptDNA contains other ESR1 mutations associated with endocrine therapy resistance. Although additional ESR1 LBD mutations have been described at lower frequency (5–9), we did not identify these mutations by NGS of tissues in the retrospective cohort, and they were therefore not queried by ddPCR. In addition, because these mutations are all in close proximity to one another, each ESR1 ddPCR mutation probe was run separately due to potential competition for the same template molecule, which could theoretically decrease the sensitivity for any given probe. This can limit the number of mutations that can be assayed because of low amounts of plasma DNA. However, this limitation may have led us to underestimate the prevalence of ESR1 mutations in our study.

In summary, we confirm the feasibility of detecting ESR1 mutations in ptDNA, and that plasma may prove to be a superior source to metastatic biopsies for ESR1 mutation detection. However, the clinical utility of using ddPCR for ESR1 mutations to guide therapy for patients requires careful prospective study before adoption into clinical practice. It is unknown what allelic frequency of ESR1 mutation is associated with symptomatic disease progression and whether changing endocrine therapies can improve patient outcomes. Nevertheless, the ability to detect ESR1 mutations in the plasma of patients, independent of the tissue mutational status, provides the foundation for future clinical trials to track and monitor the emergence of endocrine therapy resistance.

Disclosure of Potential Conflicts of Interest
D.F. Hayes reports receiving commercial grants from Janssen, commercial research support from AstraZeneca, Pfizer, and Puma; speakers bureau honoraria from Eli Lilly; has ownership interest in Onimmune and Inbiomotion; and is listed as a co-inventor on a patent regarding the novel use of CellSearch, which is owned by the University of Michigan and licensed to Janssen. B.H. Park reports receiving commercial research grants from Genomic Health, Inc.; other commercial research support from Foundation Medicine, Inc.; has ownership interest in Luxo Oncology; is a consultant/advisory board member for Luxo Oncology and Horizon Discovery. No potential conflicts of interest were disclosed by the other authors.

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Authors’ Contributions
Conception and design: D. Chu, C. Paoletti, N. Tokudome, D.F. Hayes, J.M. Rae, B.H. Park
Study supervision: N. Tokudome, D.F. Hayes, J.M. Rae, B.H. Park
Other (deployment of methodology on parallel tasks): R. Gillani

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ESR1 Mutations Detected in Circulating Tumor DNA

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