Clinical Cancer Research

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

David Chu[1], Costanza Paoletti[2], Christina Gersch[2], Dustin A. VanDenBerg[1], Daniel J. Zabransky[1], Rory L. Cochran[1], Hong Yuen Wong[1], Patricia Valda Toro[1], Justin Cidado[1], Sarah Croessmann[1], Bracha Erlanger[1], Karen Cravero[1], Kelly Kyker-Snowman[1], Berry Button[1], Heather A. Parsons[1], W. Brian Dalton[1], Riaz Gillani[1], Arielle Medford[1], Kimberly Aung[2], Nahomi Tokudome[2], Arul M. Chinnaiyan[2], Anne Schott[2], Dan Robinson[2], Karen S. Jacks[3], Josh Lauring[1], Paula J. Hurley[1], Daniel F. Hayes[2], James M. Rae[2], and Ben Ho Park[1,4]

Abstract

Purpose: Mutations in the estrogen receptor (ER)α 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; 22(4); 993–9. ©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,

[1]The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland. [2]The University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan. [3]Comprehensive Cancer Centers of Nevada, Las Vegas, Nevada. [4]The Whiting School of Engineering, Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, Maryland.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

D. Chu and C. Paoletti contributed equally to this article.

Current address for J. Cidado: Oncology iMED, AstraZeneca, Waltham, Massachusetts.

Corresponding Authors: Ben Ho Park, The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, CRB, Room 151, 1650 Orleans Street, Baltimore, MD 21287. Phone: 410-502-7399; Fax: 410-614-4073; E-mail: bpark2@jhmi.edu; and James M. Rae, Division of Hematology/Oncology, Department of Internal Medicine, University of Michigan Medical Center, 1500 East Medical Center Dr, 6310 Cancer Center, Ann Arbor, MI 48109-5942; E-mail: jimmyrae@umich.edu

doi: 10.1158/1078-0432.CCR-15-0943

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.

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 disease 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.

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 (JHSKCCC; 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 JHSKCCC 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 centrifuge 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.

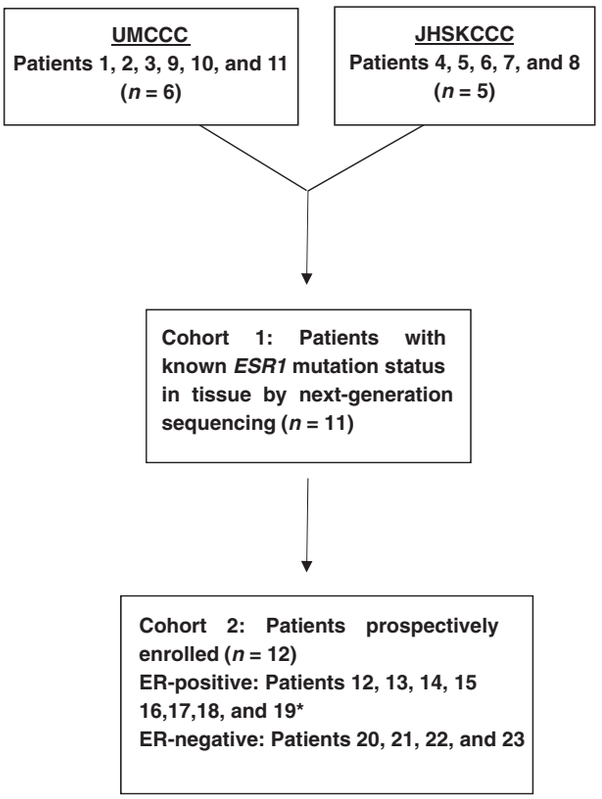

Figure 1.
Patient enrollment and distribution. Cohort 1, patients were enrolled retrospectively after NGS of a metastatic biopsy at UMCCC and JHSKCCC (cohort 1) confirming the presence of an *ESR1* mutation (patients 1, 2, 3, 4, 5, 6, 7, and 8) or wild-type *ESR1* (patients 9, 10, and 11) with blood obtained after tissue sequencing was performed for ddPCR. Cohort 2, patients were enrolled prospectively, with tissue and blood obtained contemporaneously. Tissue was subjected to NGS and blood was analyzed by ddPCR, with sequencing results blinded to ddPCR investigators. *, All cohort 2 patients were enrolled at UMCCC except patient 19.

wild-type) between tissue and blood using a 2 × 2 contingency table with 9 samples for tissue and 12 samples for blood.

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 (median, 145 days; range, 54–344 days). Eight of these patients (patients 1–8) had *ESR1* mutations identified via NGS in their metastatic biopsies, and 3 patients (patients 9–11) had wild-type *ESR1*. Patients 1 to 3 had previously been reported as having an *ESR1* tissue mutation (7) identified via the MiONCOSEQ program at UMCCC (26), whereas patients 4–8 had *ESR1* mutations identified at JHSKCCC using a commercial platform (Foundation Medicine). All patients had documented ER-positive disease, and NGS was performed on samples representing diverse metastatic sites (Table 1). The plasma specimens were interrogated for all three *ESR1* hotspot mutations: Y537S, Y537N, and D538G, using mutation-specific probes and ddPCR as previously reported (19). These three mutations were chosen as they collectively represent the most frequent *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) 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

Table 1. Patient characteristics

Patient	Age at study entry (median = 58), y	Site of tissue biopsy	Primary ER/PR/HER2	Metastatic ER/PR/HER2	Treatment
Cohort 1					
1	58	Peritoneal fluid	+/+/−	+/+/−	Tamoxifen × 5 y (adjuvant), letrozole (metastatic), tamoxifen (metastatic), fulvestrant, exemestane + everolimus
2	45	Liver	+/+/−	+/+/−	Tamoxifen × 4 y (adjuvant), anastrozole (metastatic), fulvestrant, estrace, exemestane
3	60	Liver	+/+/−	+/+/+	Tamoxifen × 5 y (adjuvant), letrozole (metastatic)
4	56	Liver	NA	+/+/−	Tamoxifen and zolendronic acid × 4 y, anastrozole × 6 wk, fulvestrant × 1 y, exemestane × 2 mo
5	37	Liver	+/−/−	+/−/−	Tamoxifen × 2 y (adjuvant), letrozole (+sorafenib on trial) × 4 mo, anastrozole × 1 y, fulvestrant x 4 mo
6	63	Brain	+/+/NA	+/+/−	Tamoxifen × 1 y, letrozole × 1 y, fulvestrant × 6 mo
7	70	Liver	+/+/NA	+/+/−	Tamoxifen (adjuvant), letrozole, exemestane + everolimus, fulvestrant (many years)
8	65	Liver	+/+/NA	+/+/−	Tamoxifen × 5 y (adjuvant), letrozole × 5 y (adjuvant), anastrozole × 1.5 y, fulvestrant × 8 mo
9	46	Sternal mass	+/+/−	+/−/−	Tamoxifen (adjuvant), anastrozole (adjuvant), anastrozole (metastatic), fulvestrant + anastrozole (metastatic)
10	67	Skin and subcutaneous tissue	+/+/−	+/+/−	Tamoxifen × 5 y (adjuvant), exemestane, fulvestrant (metastatic)
11	58	Bone	+/+/−	+/−/−	Tamoxifen × 2 y (adjuvant), anastrozole (adjuvant), fulvestrant (metastatic)
Cohort 2					
12	61	Unable to get tissue for analysis	+/NA/NA	+/+/−	Tamoxifen × 3.5 y (adjuvant), letrozole × 1.5 y (adjuvant), anastrozole (adjuvant), fulvestrant + anastrozole (metastatic)
13	63	Periaortic lymph node	+/+/−	+/+/−	Tamoxifen × 3 y (adjuvant), exemestane × 5 y (adjuvant), anastrozole (metastatic), anastrozole + fulvestrant, exemestane
14	48	Pleural fluid	+/+/+	NA	Tamoxifen (metastatic), fulvestrant + leuprolide, fulvestrant + anastrozole + leuprolide
15	56	Right axillary lymph node	+/−/−	+/NA/−	Letrozole + goserelin (neoadjuvant), letrozole + goserelin (adjuvant), fulvestrant (metastatic), fulvestrant + letrozole
16	77	Liver	+/+/+	+/−/−	Tamoxifen × 5 y, exemestane (adjuvant), anastrozole (adjuvant)
17	63	Axillary lymph node	+/−/−	NA	Anastrozole (metastatic), fulvestrant, tamoxifen, exemestane + everolimus
18	65	Subcutaneous chest wall nodule	+/−/−	+/−/−	Anastrozole (adjuvant), tamoxifen (adjuvant), fulvestrant (metastatic)
19	65	Unable to get tissue for analysis	+/+/−	NA	Letrozole × 5 y (metastatic), fulvestrant
20	63	Skin	−/−/+	−/−/+	None
21	41	Lung, right lower lobe	−/−/+	−/−/+	None
22	49	Right anterior chest wall	−/−/−	−/−/−	None
23	57	Liver	−/−/−	−/−/−	None

Abbreviation: NA, not available.

ptDNA. The difference in mutational status was statistically significant 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 amenable 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 Supplementary 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-positive metastatic disease. She initiated treatment with the aromatase inhibitor letrozole, and after 1 year of therapy, restaging scans showed disease stabilization of her bony metastasis and

Table 2. Tumor samples from 11 patients with ER-positive metastatic breast cancer were analyzed for *ESR1* mutations using NGS by sequencing FFPE tumor tissue

Patient	Days between tissue biopsy and blood draw	Sequencing FFPE tumor tissue	ddPCR plasma for *ESR1* Y537S	ddPCR plasma for *ESR1* Y537N	ddPCR plasma for *ESR1* D538G
1	186	*ESR1* Y537S	Y537S (0.87%)	Wild-type	D538G (0.01%)
2	344	*ESR1* Y537S	Y537S (1.69%)	Wild-type	Wild-type
3	275	*ESR1* D538G	Wild-type	Wild-type	D538G (1.55%)
4	68	*ESR1* Y537S	Y537S (0.63%)	Wild-type	Wild-type
5	64	*ESR1* D538G	Wild-type	Wild-type	D538G (0.03%)
6	165	*ESR1* D538G	Wild-type	Wild-type	D538G (4.23%)
7	88	*ESR1* D538G	Wild-type	Wild-type	D538G (0.01%)
8	60	*ESR1* Y537N	Wild-type	Y537N (0.68%)	Wild-type
9	54	Wild-type	Wild-type	Wild-type	D538G (0.01%)
10	145	Wild-type	Wild-type	Wild-type	Wild-type
11	270	Wild-type	Wild-type	Wild-type	Wild-type

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.

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

Table 3. Tumor samples from 8 and 4 patients with ER-positive and ER-negative metastatic breast cancer, respectively, were analyzed for *ESR1* mutations using NGS by sequencing FFPE tumor tissue

Patient	Days between tissue biopsy and blood draw	Sequencing FFPE tumor tissue	ddPCR plasma for *ESR1* Y537S	ddPCR plasma for *ESR1* Y537N	ddPCR plasma for *ESR1* D538G
ER-positive					
12	—	n/a	Y537S (0.47%)	Wild-type	Wild-type
13	0	Wild-type	Wild-type	Wild-type	D538G (0.01%)
14	0	n/a	Y537S (5.02%)	Wild-type	D538G (2.62%)
15	0	Wild-type	Wild-type	Wild-type	Wild-type
16	0	Wild-type	Wild-type	Wild-type	D538G (0.01%)
17	0	Wild-type	Wild-type	Wild-type	Wild-type
18	0	Wild-type	Wild-type	Wild-type	D538G (0.01%)
19	—	n/a	Wild-type	Y537N (0.06%)	Wild-type
ER-negative					
20	5	Wild-type	Wild-type	Wild-type	Wild-type
21	3	Wild-type	Wild-type	Wild-type	Wild-type
22	0	Wild-type	Wild-type	Wild-type	Wild-type
23	0	Wild-type	Wild-type	Wild-type	Wild-type

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 non-biopsied 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 higher 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 Astra Zeneca, Pfizer, and Puma; speakers bureau honoraria from Eli Lilly; has ownership interest in Inbiomotion and OncImmune; 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 Loxo Oncology; and is a consultant/advisory board member for Horizon Discovery and Loxo Oncology. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

None of the funding sources influenced the design, interpretation, or submission of this article.

Authors' Contributions

Conception and design: D. Chu, C. Paoletti, N. Tokudome, D.F. Hayes, J.M. Rae, B.H. Park

Development of methodology: D. Chu, C. Paoletti, C. Gersch, D.A. VanDenBerg, D.J. Zabransky, R.L. Cochran, H.Y. Wong, P.V. Toro, S. Croessmann, K. Cravero, K. Kyker-Snowman, B. Button, H.A. Parsons, W.B. Dalton, A. Medford, N. Tokudome, J. Lauring, P.J. Hurley, J.M. Rae, B.H. Park

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Chu, C. Paoletti, D.J. Zabransky, R.L. Cochran, S. Croessmann, B. Erlanger, K. Cravero, K. Kyker-Snowman, B. Button, H.A. Parsons, W.B. Dalton, K. Aung, A. Schott, D. Robinson, K.S. Jacks, J. Lauring, P.J. Hurley, J.M. Rae

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Chu, C. Paoletti, C. Gersch, D.A. VanDenBerg, D.J. Zabransky, R.L. Cochran, P.V. Toro, S. Croessmann, K. Cravero, K. Kyker-Snowman, B. Button, H.A. Parsons, W.B. Dalton, A. Medford, K. Aung, A.M. Chinnaiyan, A. Schott, D. Robinson, K.S. Jacks, J. Lauring, P.J. Hurley, D.F. Hayes, J.M. Rae, B.H. Park

Writing, review, and/or revision of the manuscript: D. Chu, C. Paoletti, C. Gersch, D.J. Zabransky, R.L. Cochran, H.Y. Wong, P.V. Toro, J. Cidado, S. Croessmann, K. Cravero, K. Kyker-Snowman, B. Button, H.A. Parsons, W.B. Dalton, R. Gillani, A. Medford, K. Aung, N. Tokudome, A.M. Chinnaiyan, A. Schott, K.S. Jacks, J. Lauring, P.J. Hurley, D.F. Hayes, J.M. Rae, B.H. Park

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Chu, C. Paoletti, D.A. VanDenBerg, R.L. Cochran, H.Y. Wong, 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

Acknowledgments

The authors thank Moshe Talpaz, MD, for assistance with the MiONCOSEQ data.

Grant Support

This work was supported in part by The Breast Cancer Research Foundation (BCRF) grant N003173 (J. Rae and D.F. Hayes), The Avon Foundation (B.H. Park, J. Lauring), DOD W81XWH-14-1-0284 (P. Hurley), NIH CA009071 (H. Parsons, K. Cravero, B.H. Park), GM007309 (D.J. Zabransky), CA168180

(R.L. Cochran), CA167939 (S. Croessmann), and the Sandy Garcia Charitable Foundation (D. Chu and B. Erlanger). The authors also thank and acknowledge the support of NIH P30 CA006973, the Commonwealth Foundation, the Santa Fe Foundation, the Breast Cancer Research Foundation (B.H. Park), the Health Network Foundation, the ME Foundation, the Augustine Fellowship (W.B. Dalton), The Walsh Fund (A. Medford), The Robin Page/Lebor Foundation and Fashion Footwear Charitable Foundation of New York/QVC Presents Shoes on Sale (D.F. Hayes).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 17, 2015; revised July 6, 2015; accepted July 28, 2015; published OnlineFirst August 10, 2015.

References

1. Osborne CK. Tamoxifen in the treatment of breast cancer. N Engl J Med 1998;339:1609–18.
2. Burstein HJ, Prestrud AA, Seidenfeld J, Anderson H, Buchholz TA, Davidson NE, et al. American Society of Clinical Oncology clinical practice guideline: update on adjuvant endocrine therapy for women with hormone receptor-positive breast cancer. J Clin Oncol 2010;28:3784–96.
3. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
4. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med 2011;62:233–47.
5. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. Cell Rep 2013;4:1116–30.
6. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, et al. D538G mutation in estrogen receptor-alpha: a novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res 2013;73:6856–64.
7. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet 2013;45:1446–51.
8. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet 2013; 45:1439–45.
9. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res 2014;20:1757–67.
10. Zhang QX, Borg A, Wolf DM, Oesterreich S, Fuqua SA. An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. Cancer Res 1997;57:1244–9.
11. Weis KE, Ekena K, Thomas JA, Lazennec G, Katzenellenbogen BS. Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. Mol Endocrinol 1996; 10:1388–98.
12. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
13. Leary RJ, Kinde I, Diehl F, Schmidt K, Clouser C, Duncan C, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2010;2:20ra14.
14. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res 2012;18:3462–9.
15. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4:162ra54.
16. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4:136ra68.
17. Chan KC, Jiang P, Chan CW, Sun K, Wong J, Hui EP, et al. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proc Natl Acad Sci U S A 2013;110:18761–8.
18. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–12.
19. Beaver JA, Jelovac D, Balukrishna S, Cochran RL, Croessmann S, Zabransky DJ, et al. Detection of cancer DNA in plasma of patients with early-stage breast cancer. Clin Cancer Res 2014;20:2643–50.
20. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.
21. Stroun M, Maurice P, Vasioukhin V, Lyautey J, Lederrey C, Lefort F, et al. The origin and mechanism of circulating DNA. Ann N Y Acad Sci 2000; 906:161–8.
22. Choi JJ, Reich CF III, Pisetsky DS. The role of macrophages in the in vitro generation of extracellular DNA from apoptotic and necrotic cells. Immunology 2005;115:55–62.
23. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008; 14:985–90.
24. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486:537–40.
25. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 2012;486:532–6.
26. Roychowdhury S, Iyer MK, Robinson DR, Lonigro RJ, Wu YM, Cao X, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study. Sci Transl Med 2011;3:111ra21.
27. Oesterreich S, Davidson NE. The search for ESR1 mutations in breast cancer. Nat Genet 2013;45:1415–6.