

# Prevalence of *ESR1* Mutations in Cell-Free DNA and Outcomes in Metastatic Breast Cancer

## A Secondary Analysis of the BOLERO-2 Clinical Trial

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**IMPORTANCE** Estrogen receptor  $\alpha$  (*ESR1*) mutations found in metastatic breast cancer (MBC) promote ligand-independent receptor activation and resistance to estrogen-deprivation therapy in laboratory models. The prevalence of these mutations and their potential impact on clinical outcomes has not been established.

**OBJECTIVE** To determine the prevalence of *ESR1* mutations (Y537S and D538G) in estrogen receptor (ER)-positive MBC and determine whether mutation is associated with inferior outcomes.

**DESIGN, SETTING, AND PARTICIPANTS** From December 16, 2014, to August 26, 2015, we analyzed cell-free DNA (cfDNA) from baseline plasma samples from participants in the BOLERO-2 double-blind phase 3 study that randomized patients from 189 centers in 24 countries with MBC to exemestane plus placebo or exemestane plus everolimus. The study enrolled postmenopausal women with a diagnosis of MBC and prior exposure to an aromatase inhibitor. Baseline plasma samples were available from 541 of 724 patients (74.7%). We assessed the effect of mutation on overall survival of the population and the effect of mutation on progression-free survival (PFS) by treatment arm.

**INTERVENTIONS** Patients were randomized to treatment with exemestane (25 mg oral daily) together with everolimus (10 mg oral daily) or with placebo.

**MAIN OUTCOMES AND MEASURES** The 2 most frequent mutations in *ESR1* (Y537S and D538G) were analyzed from cfDNA using droplet digital polymerase chain reaction and samples scored as wild-type, D538G, Y537S, or double mutant. Cox-proportional hazards model was used to assess PFS in patient subgroups defined by mutations, and the effect of each mutation on overall survival.

**RESULTS** Of 541 evaluable patients, 156 (28.8%) had *ESR1* mutation D538G (21.1%) and/or Y537S (13.3%), and 30 had both. These mutations were associated with shorter overall survival (wild-type, 32.1 months [95% CI, 28.09-36.40 months]; D538G, 25.99 months [95% CI, 19.19-32.36 months]; Y537S, 19.98 months [13.01-29.31 months]; both mutations, 15.15 months [95% CI, 10.87-27.43 months]). The D538G group (hazard ratio, 0.34 [95% CI, 0.02-0.57]) derived a similar PFS benefit as wild type from addition of everolimus to exemestane.

**CONCLUSIONS AND RELEVANCE** *ESR1* mutations are prevalent in ER-positive aromatase inhibitor-treated MBC. Both Y537S and D538G mutations are associated with more aggressive disease biology.

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Activation of the estrogen receptor (ER) is a key feature of the 70% to 80% of breast cancers in which ER expression is detected.<sup>1</sup> Estrogen deprivation therapy in the form of an aromatase inhibitor (AI) is an effective, targeted therapy for these tumors reducing disease morbidity and mortality. Outcomes of patients with ER-positive metastatic breast cancer (MBC) who are treated with AIs vary considerably, with tumor relapse occurring within months in some patients and only after many years in others.<sup>2</sup> Constitutively active mutation in the ER has recently been identified as a recurrent event in ER-positive MBC, although their prevalence is not well established.<sup>3-5</sup> These mutations are observed in the ligand binding domain and promote the receptors adopting an active conformation even in the absence of ligand. It has been speculated that these mutations may reduce the efficacy of hormonal therapies such as AIs; however, evidence of their impact on clinical outcomes is lacking. We thus investigated the effect of *ESR1* mutation on disease outcomes for the BOLERO-2 clinical trial.<sup>6</sup> Briefly, BOLERO-2 was a double-blind, randomized, placebo-controlled phase 3 trial that compared the AI, exemestane, with the combination of exemestane and the mammalian target of rapamycin complex 1 (mTORC1) inhibitor, everolimus, in patients with hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-negative MBC that had progressed following treatment with a nonsteroidal AI. Improvement in progression-free survival (PFS) was associated with everolimus (median PFS, 7.8 months compared with 3.2 months; hazard ratio [HR], 0.45; 95% CI, 0.38-0.54) across all patient subgroups, including an assessment of common tumor genetic aberrations.<sup>6,15</sup>

## Methods

### Study Design and Patients

The complete study design and outcomes for the BOLERO-2 clinical trial were reported previously.<sup>6,7</sup> Patients provided written informed consent to the evaluation of their plasma samples in the present analysis of cell-free DNA (cfDNA). They were not compensated for their participation. Consent for participation in the clinical trial and use of plasma samples was approved by each local institutional review board (ethical review of study by the local institutional review board was waived per Department of Health and Human Services regulation 45 CFR Part 46-E4). Samples were analyzed from December 16, 2014, to August 26, 2015.

### Plasma Processing

Baseline blood samples were obtained in EDTA tubes and plasma prepared within 30 minutes of collection by centrifugation (1100-1300g). Plasma was aliquoted and stored at  $-70^{\circ}\text{C}$  (or  $-20^{\circ}\text{C}$  if not available). Immediately after thawing, all plasma samples underwent a high speed centrifugation (16 000g) followed by DNA extraction. The volume of cleared plasma used to extract cfDNA ranged from 0.3 to 3.3 mL with a median of 1.8 mL. To assess the feasibility of analyzing

## Key Points

**Question** What is the prevalence and clinical significance of estrogen receptor mutation in patients with metastatic breast cancer?

**Findings** In a secondary analysis of cell-free DNA from 541 patients enrolled in a randomized clinical trial, 29% of patients (156) had a mutation in the estrogen receptor. Patients with mutation had a significantly shorter median overall survival (20.7 months) compared with those without mutation (32.1 months).

**Meaning** Mutations in the estrogen receptor are common in patients with metastatic breast cancer who were previously treated with an aromatase inhibitor and are associated with worse outcomes.

small-volume archival frozen plasma samples, cfDNA was manually extracted ( $N = 248$ ) by manufacturer's protocol using QIAamp Circulating Nucleic Acid Kit (Qiagen Inc). The concentration and integrity of cfDNA was determined by quantitative polymerase chain reaction (PCR) using the KAPA Human Genomic DNA quantification and QC kit (KAPABio-systems). Following confirmation that archival cfDNA was informative for droplet digital PCR (ddPCR) analysis, the remaining samples underwent semi-automated extraction with QIASymphony SP system and DSP Virus/Pathogen Kit (Qiagen Inc).

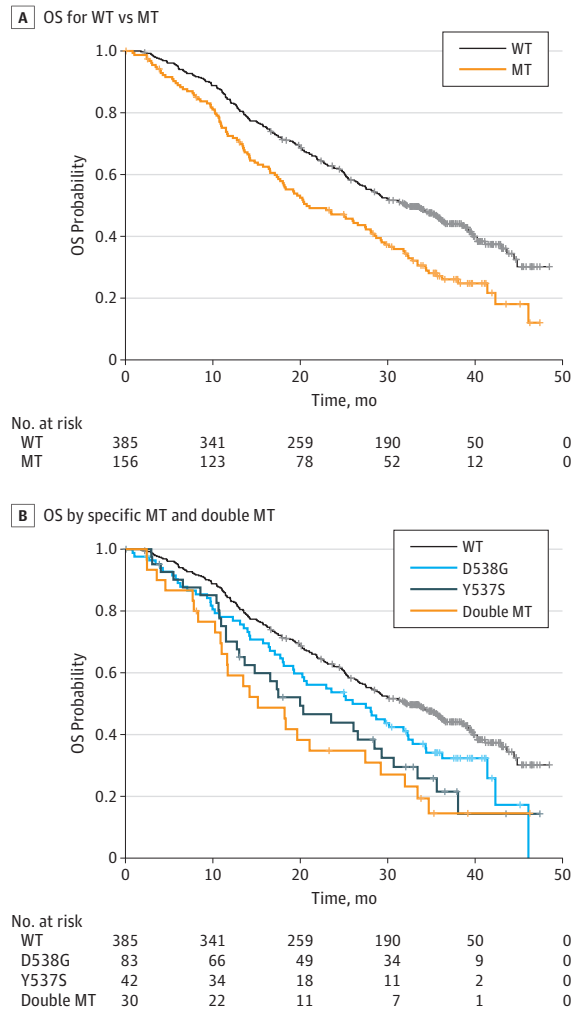
### *ESR1* Mutation Detection and Quantification

Allele-specific assays for *ESR1* D538G and Y537S mutations were designed and optimized for quantification on a BioRad QX200 ddPCR System (BioRad) similar to prior reports.<sup>8,9</sup> Oligonucleotide sequence for primers and probes and ddPCR reaction conditions are noted in supplementary materials (eTable 1 in the Supplement). Validation of the assays was performed with genomic tumor DNA mutated for *ESR1*, negative wild-type genomic DNA, and no template controls. No cross-reaction activity was identified. The extracted cfDNA was analyzed for the presence of the *ESR1* D538G and Y537S mutations by ddPCR in singleplex assays that included mutation positive, wild-type template, and no template controls. All assays were manually reviewed to assess amplitude threshold and clear separation of positive and negative partitions; a sample was determined positive for mutation if a 6-fluorescein amidite (6-FAM)-positive droplet was detected. Mutation analyses were performed on anonymized samples, and data were transferred to Novartis Web Data Communication System for statistical analysis.

### Statistical Analysis

Cox proportional hazards models were used to assess the prognostic and predictive effects of *ESR1* mutations on PFS and overall survival (OS). To assess the predictive effects of mutations on PFS, hazard ratios (HRs) with 95% CIs were calculated for treatment vs placebo arms in the wild-type and mutant subgroups. To assess the prognostic effects of mutations on OS, HRs with 95% CIs were calculated for the wild-type vs mutant subgroups. Multivariate analysis was also

Figure 1. Kaplan-Meier Curves for Overall Survival (OS) According to Mutation Status



A, Superior overall survival (OS) is shown for patients without mutation (MT) in *ESR1* (wild-type [WT]) compared with those with D538G and/or Y537S mutation. B, Overall survival results are shown for WT or D538G alone or Y537S alone or both D538G and Y537S (double MT).

performed to adjust for the potential effects of prior hormonal therapy, visceral disease, and ECOG status. *ESR1* mutation frequencies in subgroups defined by baseline clinical covariates were calculated, and Pearson-Klopper method was used to compute the 95% CIs of the frequencies.  $\chi^2$  Tests were used to demonstrate whether there were differences in *ESR1* mutation frequencies between those subgroups. All 2-sided *P* values were nominal without adjustment for multiple testing. All analyses were hypothesis-driven; *P* < .05 was considered statistically significant. The PFS data are based on the data cutoff of December 2011, and OS data are based on the data cutoff of October 2013. All analyses were performed using SAS statistical software (version 9.3) or R statistical software (version R2.15.2).

## Results

### ESR1 Mutation Prevalence and Associations With Clinical Characteristics

Cell-free DNA was evaluable for *ESR1* from 541 of the 724 patients (74.7%) enrolled in the BOLERO-2 clinical trial (eFigure 1 in the Supplement). Lack of consent to genomic testing was the major reason for excluding cfDNA analysis for the remaining 162 patients. The median total cfDNA for the sample population was 14.4 ng (minimum, 2.4 ng; maximum, 1130.0 ng). An *ESR1* mutation (Y537S and/or D538G) was detected in 28.8% of samples (156 of 541), D538G was the more prevalent mutation occurring in 114 patients (21.1%), whereas Y537S occurred in 72 patients (13.3%), and both mutations were identified in 30 patients (eTable 2 in the Supplement).

Among baseline clinical covariates, there was a moderate difference (*P* = .04) in the frequency of *ESR1* mutation by ECOG status but not by age, race, sites of metastatic disease, or sensitivity to prior hormone therapy (eTable 3 in the Supplement). A notable difference associated with mutation prevalence was line of therapy. There was a 3-fold increase in mutation prevalence in patients who had failed first-line therapy for metastatic disease (33% were mutant) compared with those who were initiating first-line treatment for MBC (11% were mutant), in whom exposure to AI therapy occurred only in the adjuvant setting (eTable 4 in the Supplement). The finding is consistent with data from a small series that was very recently reported.<sup>9</sup>

### Overall Survival

The D538G and Y537S *ESR1* mutations promote constitutive biochemical activation of the receptor, which is a well-known driver of breast cancer growth.<sup>3</sup> The high prevalence of these alterations led us to ask whether these mutations are associated with less favorable outcomes in this cohort of patients with MBC previously exposed to aromatase inhibition. We compared the overall outcomes of the cfDNA population and the intention-to-treat population and found them to be highly concordant (eTable 5 in the Supplement). Patients with neither D538G nor Y537S mutation had a median OS of 32.1 months (95% CI, 28.09-36.4 months), compared with 20.73 months (95% CI, 17.71-28.06 months) for patients with mutation (Figure 1A). The effect of individual mutation also had an impact, with a median OS of 25.99 months (95% CI, 19.19-32.36 months) for patients with only D538G mutation and 19.98 months (95% CI, 13.01-29.31 months) for patients with only Y537S mutation (Figure 1B). Notably, patients having both mutations had a further reduced median OS (15.15 months; 95% CI, 10.87-27.43 months). These data identify a poor prognostic association with these 2 prevalent mutations on OS that is confirmed by multivariate analysis for the MT, Y537S, and double MT groups (Table 1).

### Progression-Free Survival

While the Y537S and D538G mutations both result in biochemical activation of the receptor in the absence of hor-

Table 1. Overall Survival (OS) by Mutation and Multivariate Analysis<sup>a</sup>

Group	No.	Events	OS, Median (95% CI), mo	Hazard Ratio (95% CI)		P Value <sup>b</sup>
				Univariate	Multivariate	
Wild-type	385	217	32.1 (28.09-36.40)	NA	NA	NA
Mutated	156	112	20.73 (17.71-28.06)	1.62 (1.29-2.03)	1.59 (1.26-2.00)	<.001
D538G	83	57	25.99 (19.19-32.36)	1.39 (1.04-1.86)	1.32 (0.98-1.76)	.03
Y537S	42	30	19.98 (13.01-29.31)	1.80 (1.23-2.63)	1.85 (1.26-2.72)	.003
Double mutated	30	24	15.15 (10.87-27.43)	2.23 (1.46-3.40)	2.39 (1.56-3.65)	<.001

Abbreviation: NA, not applicable.

hormonal therapy, visceral disease, and ECOG status.

<sup>a</sup> Multivariate analysis was performed to adjust for the potential effects of prior<sup>b</sup> P values are from univariate analysis without adjusting for clinical covariates.

Table 2. Impact of ESR1 Mutation on Progression-Free Survival With Exemestane Therapy Alone

Alteration	No.	Events	PFS, Median (95% CI), mo	HR (95% CI)	P Value
Wild-type	128	116	3.94 (2.76-4.17)	NA	NA
Mutated	61	51	2.76 (1.41-4.14)	1.27 (0.91-1.77)	.16
D538G	24	22	2.69 (1.35-2.83)	1.71 (1.09-2.68)	.02
Y537S	21	16	4.14 (1.38-6.70)	0.95 (0.56-1.61)	.86
Double mutated	15	12	2.78 (1.41-6.87)	1.21 (0.67-2.21)	.53

Abbreviations: HR, hazard ratio; NA, not applicable.

mone, the manner in which these mutations alter receptor function and thus influence cancer cell behavior may differ. We examined the effect of each mutation upon PFS in both the placebo (exemestane) and treatment (everolimus) arms of the study. In the exemestane arm, mutation only in D538G was associated with shorter PFS (2.69 months; HR, 1.71; 95% CI, 1.09-2.68 months) compared with wild-type (3.94 months) (Table 2). Mutation in Y537S did not show a difference from wild-type, albeit with fewer cases (4.14 months; HR, 0.95; 95% CI, 0.56-1.61 months) (eFigure 2 in the Supplement).

In terms of the improvement in PFS with the addition of everolimus, patients with both wild-type (8.48 months; HR, 0.4; 95% CI, 0.31-0.51 months) and D538G mutation (5.78 months; HR, 0.34; 95% CI, 0.02-0.57 months) showed a significant improvement (Figure 2A; eTable 6 in the Supplement). The number of cases of Y537S in each arm was small, and so it was not possible to definitively assess everolimus benefit for this subgroup. It is hypothesis-generating that among the cases present in this series, everolimus benefit was not apparent for Y537S alone (4.17 months; HR, 0.98; 95% CI, 0.49-1.94 months) or cases with both D538G and Y537S (5.42 months; HR, 0.53; 95% CI, 0.23-1.25 months) (Figure 2B; eTable 6 in the Supplement).

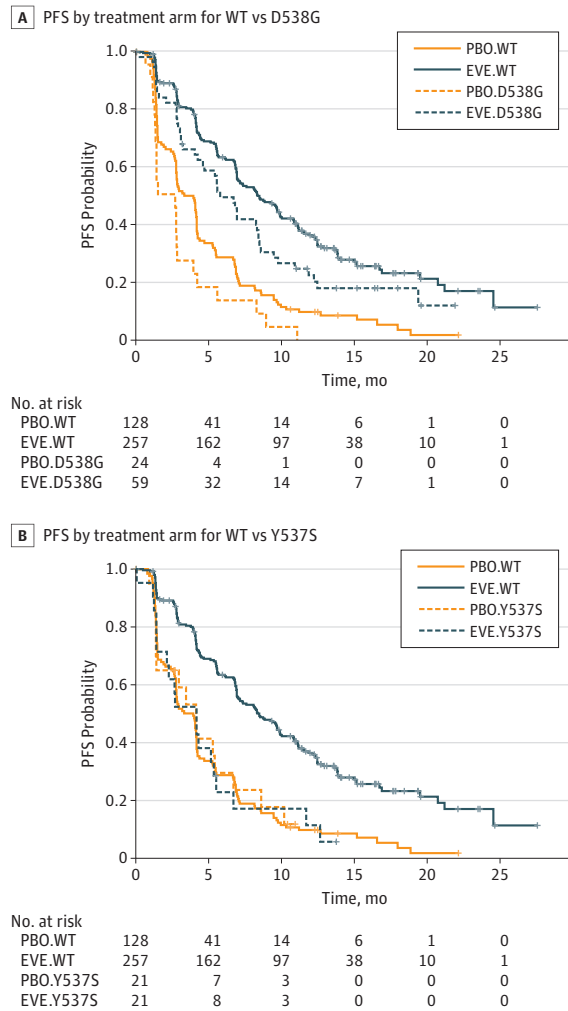
## Discussion

Although metastatic breast cancer is an incurable and lethal disease, patients with ER-positive MBC have highly variable clinical courses. In many cases, hormonal therapy may be given as single agents for many years with outstanding disease control and limited adverse effects. In other cases, hormonal therapy has more limited benefit and the addition of further agents is likely needed to achieve durable disease control. Presently, there is little to no guidance on which patients may fall

into the former or latter group. This is unlike the situation in primary breast cancer where a host of prognostic and predictive tools have been developed to stratify the clinical utility of adjuvant systemic therapies.<sup>10,11</sup> In the present study, we asked whether mutations in the key driver of ER-positive breast cancer, the estrogen receptor, might serve as a tool to better define this large population of patients with cancer for whom an increasing menu of therapy choices are available. In this series, we found that 2 ESR1 mutations, Y537S and D538G, were highly prevalent in ER-positive MBC and associated with inferior outcomes.

One of the key findings of this work is the high prevalence of ER mutations in this patient population with AI-treated, ER-positive MBC. ESR1 mutations were initially identified in small series of patients with most mutations identified in metastatic samples often from heavily treated patients.<sup>3-5</sup> Thus, an accurate measurement of mutation prevalence was lacking. Recent work has suggested that cfDNA may be an effective source to look for these alterations enabling easier sampling from a common time point, such as entry onto a large clinical trial.<sup>12-14</sup> Indeed, we previously noted a lower prevalence of ESR1 mutation by tumor sequencing, which likely relates to archival primary tumors being used for these analyses.<sup>3,15</sup> Using cfDNA collected at the time of study entry, we found that nearly 30% of patients with ER-positive MBC have ESR1 mutations D538G and/or Y537S. Interestingly, although all patients enrolled on the BOLERO-2 trial had prior exposure to AI, there were some who received in the adjuvant setting while most received it in the metastatic setting. Our data find the mutation prevalence varies between these groups, with mutation identified in 11% of patients who had received AI therapy only in the adjuvant setting compared with 33% in patients who had previously received AI for treatment for MBC. These differences in prevalence further substantiate genomic evolution in metastatic ER-positive breast cancer and imply a potential value for active genomic testing in the clinic.

Figure 2. Kaplan-Meier Curves for Effect of Addition of Everolimus to Exemestane



A, Progression-free survival (PFS) for patients without *ESR1* mutation (wild-type [WT]) or with the D538G mutation (D538G). B, Results for patients without *ESR1* mutation (WT) or with the Y537S mutation (Y537S). Addition of everolimus (EVE) was associated with improved progression-free survival (PFS) for patients with WT or D538G mutation but not for those with Y537S mutation. PBO indicates placebo.

The high prevalence of these mutations, along with their biologic relevance to ER-driven tumors, led us to investigate whether they might serve as biomarkers for disease outcomes. Specifically, we investigated whether these mutations might be associated with a shorter survival time. This proved to be the case with detection of any *ESR1* mutation, the D538G mutation alone, the Y537S mutation alone, or both mutations being associated with decreases in median OS. This raises the possibility that tumors with only wild-type *ESR1* may comprise a particularly favorable subgroup. Moreover, the “wild-type” designated subgroup is likely to have a small number of patients with mutation in *ESR1* other than Y537S or D538G. While these are the 2 most frequent alterations observed in tumor sequencing projects, additional alterations,

such as E380Q, Y537N, Y537C, and L536R, are also seen and would not be detected in our data set. A corollary to incomplete *ESR1* interrogation is that the current D538G cohort may include a few patients with both D538G and additional mutations. In our series, the outcomes data on the few patients with both D538G and Y537S demonstrated that this group had the shortest OS. In addition, the wild-type subgroup very likely includes a subset of patients who would develop an *ESR1* mutation over the course of their therapy on study. This is an issue with any static biomarker being used in a dynamic disease. Despite these limitations, the presence of these 2 mutations at the time of study enrollment shows a clear and strong association with shorter survival.

Naturally, we sought to use this exploratory analysis to determine whether *ESR1* mutations may serve as predictive biomarkers for aromatase and/or mTORC1 inhibition. In this case, the interaction seems more complex. Lacking a study arm that received no therapy, it was not possible to definitively determine if *ESR1* mutation predicted for lack of response to exemestane. Moreover, the group of patients under study all had prior exposure to AIs and so various other mechanisms of resistance may have developed in the wild-type population, leading to their modest PFS duration of 4 months. However, there was a decrease in PFS for the D538G mutant population compared with wild-type in the exemestane arm, so it remains plausible that the mutation may have predictive power.

With respect to the benefit of everolimus, we confirmed that most patients benefit from the addition of this drug. Both the wild-type and mutant groups had a demonstrated increase in PFS from the addition of everolimus. By mutation site, this benefit was also evident for the D538G subgroup. The study lacked sufficient numbers of patients with Y537S to draw conclusions on everolimus benefit for this subgroup. Moreover, our preliminary data showed a potential lack of benefit in those with either the Y537S mutation alone or with both Y537S and D538G. An interaction between this specific allele and mTOR activity has not been biologically identified. Thus, this particular data are hypothesis generating and ought to spark further biological and clinical investigation into potential *ESR1* mutant allele-specific effects. Overall, as a large suite of drugs (CDK4/6 inhibitors, HDAC inhibitors, PI3K inhibitors, new ER antagonists) are being developed for ER-positive MBC, assessment of *ESR1* mutation status may prove to be a valuable predictive biomarker and ought to be incorporated in these studies.

A final point about these findings was the ease and feasibility with which this biomarker was able to be obtained. The plasma samples were collected worldwide and not specifically handled with the intention of analyzing cfDNA; ddPCR assays are far from cumbersome and would be facile to implement in clinical practice. With this information, clinicians and investigators facing a wide range of outcomes may identify clinically valuable information regarding prognosis and prediction about treatments under consideration. The ease and affordability of such a test will also enable dynamic testing that will improve our understanding of the evolution of this disease and the design of strategies to improve outcomes.

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**Study concept and design:** Chandralapaty, Chen, Gnant, Baselga, Moynahan.

**Acquisition, analysis, or interpretation of data:** Chandralapaty, Chen, He, Sung, Samoila, You, Bhatt, Patel, Voi, Hortobagyi, Baselga, Moynahan.

**Drafting of the manuscript:** Chandralapaty, He, You, Bhatt, Moynahan.

**Critical revision of the manuscript for important intellectual content:** Chandralapaty, Chen, He, Sung, Samoila, Patel, Voi, Gnant, Hortobagyi, Baselga, Moynahan.

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**Study supervision:** Chandralapaty, Chen, Gnant, Hortobagyi, Moynahan.

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## Invited Commentary

## ESR1 Mutations in Cell-Free DNA of Breast Cancer Predictive "Tip of the Iceberg"

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In this issue of *JAMA Oncology*, Chandralapaty and colleagues<sup>1</sup> report a correlative analysis of the 2 most frequent hormone-binding domain estrogen receptor (ESR1) mutations (Y537S and D538G) in hormone receptor-positive, HER2-negative advanced breast cancer using archival tumor specimens from the BOLERO-2 trial<sup>2</sup> to identify correlations between these mutations and efficacy of the mammalian target of rapamycin complex 1 (mTORC1) inhibitor everolimus. The BOLERO-2 trial<sup>2</sup> was a double-blind, pla-



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cebo-controlled, phase 3 trial of patients who recurred and/or progressed during therapy with nonsteroidal aromatase inhibitors (AIs), who were randomized to everolimus plus the steroidal AI exemestane or exemestane only, and the trial demonstrated that everolimus plus exemestane substantially improved progression-free survival (PFS). Using next-generation sequencing, approximately 5% of evaluable samples (most of those analyzed were primary tumors) from patients in BOLERO-2, compared with 2% of primary tumors from The Cancer Genome Atlas (TCGA) study, contained ESR1

mutations. The BOLERO-2 trial<sup>2</sup> was a double-blind, placebo-controlled, phase 3 trial of patients who recurred and/or progressed during therapy with nonsteroidal aromatase inhibitors (AIs), who were randomized to everolimus plus the steroidal AI exemestane or exemestane only, and the trial demonstrated that everolimus plus exemestane substantially improved progression-free survival (PFS). Using next-generation sequencing, approximately 5% of evaluable samples (most of those analyzed were primary tumors) from patients in BOLERO-2, compared with 2% of primary tumors from The Cancer Genome Atlas (TCGA) study, contained ESR1