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REVIEW

The Impact of *ESR1* Mutations on the Treatment of Metastatic Breast Cancer

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Abstract After nearly 20 years of research, it is now established that mutations within the estrogen receptor (ER) gene, *ESR1*, frequently occur in metastatic breast cancer and influence response to hormone therapy. Though early studies presented differing results, sensitive sequencing techniques now show that *ESR1* mutations occur at a frequency between 20 and 40% depending on the assay method. Recent studies have focused on several "hot spot mutations," a cluster of mutations found in the hormone-binding domain of the *ESR1* gene. Throughout the course of treatment, tumor evolution can occur, and *ESR1* mutations emerge and become enriched in the metastatic setting. Sensitive techniques to continually monitor mutant burden in vivo are needed to effectively treat patients with mutant *ESR1*. The full impact of these mutations on tumor response to different therapies remains to be determined. However, recent studies indicate that mutant-bearing tumors may be less responsive to specific hormonal therapies, and suggest that aromatase inhibitor (AI) therapy may select for the emergence of *ESR1* mutations. Additionally, different mutations may respond discretely to targeted therapies. The need for more preclinical mechanistic studies on *ESR1* mutations and the development of better agents to target these mutations are urgently needed. In the future, sequential monitoring of *ESR1* mutational status will likely direct personalized therapeutic regimens appropriate to each tumor's unique mutational landscape.

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Endocrine Resistance

Hormone receptor-positive disease is the most common presentation of breast cancer. The antiestrogens tamoxifen (Tam) and fulvestrant (Ful), along with aromatase inhibitors (AIs), are the most frequently prescribed hormonal agents targeting the ER, with excellent palliation in the metastatic setting and long-term delay of first recurrence with adjuvant therapy. However, despite improvements in the efficacy of targeted endocrine therapies following the introduction of the AIs and extended durations of Tam/AI therapy, the development of endocrine resistance remains a major cause of first recurrence and mortality in ER-positive patients. It is unlikely that complete loss of ER signaling is the driving force for the majority of endocrine resistance, as both metastatic tumors and breast cancer cell lines with acquired Tam resistance frequently retain ER expression [1–3] and can remain responsive to second or third line hormonal therapy.

ER-positive tumors do not consistently respond to hormonal therapy and have considerable heterogeneity in response to therapeutic agents. Notably, tumors can be refractory to one type of treatment (for example Tam or a nonsteroidal AI) but sensitive to another type (steroidal AI) [4]. Thus, ER-expressing tumors are not a homogenous group, even within the well-defined luminal A or B molecular subtypes. Unfortunately, these molecular subtype classifications do not adequately predict the heterogeneous response of ER-positive tumors. Thus, optimal selection of endocrine agents will require a better understanding of the mechanisms associated with the evolution of hormone resistance and the adaptation of individual tumors to the selective pressure of therapeutics. As mechanisms of resistance are elucidated, selection of patients for specific hormonal agents may be based on a combination of personalized genomics and adaptive pathway biomarkers which predict response, rather than clinical criteria alone.

The recent ESO-ESMO consensus guidelines for advanced breast cancer define acquired endocrine resistance as a relapse after the first 2 years of adjuvant endocrine treatment, a relapse within 12 months of completing adjuvant endocrine treatment, or progressive disease greater than 6 months after initiating endocrine therapy for metastatic breast cancer (MBC) [5, 6]. Unfortunately, patients with acquired resistance have a low probability of responding to further endocrine treatments for extended periods. Thus, MBC remains incurable, with median 5-year survival rates of less than 25%. Undoubtedly, endocrine resistance is common and an important clinical problem in MBC. The main identified mechanisms of endocrine resistance are related to the upregulation of escape survival pathways, such as the HER2 growth factor receptor family, and the PI3K/Akt/mammalian target of rapamycin (mTOR) pathways. However, in the past few years, there have been a dramatic change in the understanding of endocrine resistance and a general acceptance of the "rediscovery" that *ESR1* mutations and alterations (amplifications and translocations) are a central mechanism of resistance [7, 8]. This review will focus on *ESR1* alterations as a leading mechanism of intrinsic and acquired resistance in ER-positive advanced breast cancer.

Preclinical Models of Resistance

One of the first preclinical models of hormone resistance was developed by Craig Jordan and colleagues by growing MCF-7 breast cancer cells in athymic nude mice with estrogen supplementation [9]. Withdrawal of estrogen and long-term treatment of these xenograft tumors with Tam resulted in the eventual outgrowth of Tam-stimulated tumors [10]. These Tam-stimulated tumors could be serially retransplanted and displayed a stable resistant phenotype. One of several transplanted tumor lines was found to contain a single D351Y *ESR1* mutation whose transcriptional activity was indeed stimulated by Tam treatment [11]. Subsequent studies demonstrated the structural basis for the switch to estrogen-like action of Tam by substitutions at this critical residue [12]. Although this specific *ESR1* mutation has not been found in clinical samples, these studies underscore that temporal evolution of *ESR1* mutations might occur under the selective pressure of antiestrogen treatment. However, as the majority of Tam-stimulated lines contained wild-type (WT) ER, *ESR1* mutations are not thought to be the major mechanism for the agonist activity of Tam in this model.

A number of breast cancer sublines with acquired Tam resistance have been developed, the majority of which exhibit increases in growth factor receptor and subsequent downstream signaling, and in some cases, increased ER levels [13–16]. From these studies, investigators concluded that mechanisms other than alterations in ER must be important for the development of Tam-resistant growth. However, to our knowledge, reports of the presence of *ESR1* mutations in Tam-resistant sublines is limited. Based on recent findings in detecting frequent *ESR1* mutations in metastatic tumors arising after multiple lines of endocrine therapy (discussed below), deep genomic sequencing of *ESR1* in sublines with acquired resistance is likely warranted.

Sublines mimicking AI resistance have also been generated via several techniques: long-term adaptation to estrogen withdrawal [17], transfection of ER-positive cells with aromatase enzyme and maintenance in vitro [18], or grown in vivo as tumor xenografts in mice [19, 20]. Similar to that seen in Tam-resistant lines, AI resistance was accompanied by activation of growth factor receptors (EGFR and IGF-1R), the PI3K/Akt/MAPK, and mTOR pathways. Enhanced estrogen hypersensitivity was inherent in many of the lines maintained in the absence of estrogen and demonstrates that cells adapt to estrogen deprivation during AI treatment by activation of alternate signaling pathways leading to ligand-independent activation of ER [21]. These preclinical studies were some of the first to demonstrate that growth factor signaling is an important contributor to the development of endocrine resistance, and suggest that the use of signal transduction inhibitors will provide a promising alternative therapeutic strategy. However, we now know that breast cancer cells are clearly able to continue evolving and harness additional bypass pathways for growth and cell survival in the presence of signal transduction inhibitor monotherapy, and therefore, the efficacy of these inhibitors is also limited by rapid acquired resistance. The co-targeting of these adaptive pathways, along with ER signaling antagonism, is a current clinical approach under investigation [22]. Indeed, since many of the growth factor receptor pathways ultimately activate the downstream mTOR complex, mTOR has proven a promising and efficacious clinical target to restore hormone sensitivity in AI-resistant MBC [23–25].

Genomic Characterization of Breast Cancer-Derived Xenograft Models Reveals Multiple *ESR1* Alterations

The genomic landscape of primary breast cancers has recently been thoroughly explored using next generation sequencing (NGS) [26, 27]. However, the genomic basis of metastatic and endocrine-resistant breast cancer remains poorly understood. The molecular differences between primary and metastatic tumors have been a controversial area, but some studies have demonstrated that a subset of primary tumors have inherent expression signatures found in metastatic tumors, and maintain these distinct features during tumor progression [28, 29]. However, the prevailing model of metastasis holds that most primary tumor cells have low metastatic potential and that rare cells within the primary tumor acquire metastatic capacities through somatic mutation events which are selected for during tumor dissemination [30, 31].

Li et al. took a pioneering approach to examine ER-positive, endocrine-resistant tumors by establishing patient-

derived xenograft (PDX) models, which appear to predominantly maintain the hormone response profile of the originating patient tumor [32]. Using deep whole-genome NGS techniques, these ER-positive PDXs were found to contain *ESR1* hormone-binding domain (HBD) mutations (Y537S and E380D), *ESR1* amplification, and *ESR1* translocations (*ESR1*/YAP1). Most of the *ESR1* alterations were present in the original patient material; however, one mutation (E380D) arose during serial passage of the PDX models, showing that tumors can continue to evolve when maintained as xenografts in mice. Two additional *ESR1* translocations, *ESR1*/AKAP12 and *ESR1*/POLH, were reported in The Cancer Genome Atlas (TCGA) analysis of primary tumors [33]. A recurrent *ESR1*/CCDC170 fusion associated with the luminal B molecular subtype has been reported to also engage growth factor receptor signaling which reduces endocrine sensitivity [34]. Although at present these gain-of-function *ESR1* translocations are relatively infrequent (< 5%), they could play a significant role in the emergence of aggressive subpopulations in progressing tumors.

The long-term, estrogen-deprived MCF-7 model undergoes widespread genomic changes during adaptation to estrogen withdrawal, including amplification of the *ESR1* locus with subsequent increases in ER protein levels [35]. Whether *ESR1* gene amplification occurs in breast tumors, though, has been a controversial question. Holst et al. reported frequent *ESR1* amplification in 21% of breast tumors using fluorescent in situ hybridization (FISH). Amplification was associated with a significantly longer survival in Tam-treated patients [36]. This finding has been confirmed in another retrospective patient study [37]. In contrast, another study found that amplification was associated with poor disease-free and overall survival in Tam-treated patients [38], but it was found in only 1% of tumors using a variety of array hybridization platforms [39]. These discrepancies may be related to different technical protocols. Moelans et al. recently employed an RNase FISH protocol with multiplex ligation-dependent probe amplification to demonstrate that the FISH signals being interpreted as *ESR1* amplification were sensitive to RNase treatment, indicating that FISH was detecting accumulation of *ESR1* transcripts in cells expressing high levels of ER RNA, rather than gene amplification [40]. Thus, although it is apparent that *ESR1* gene amplification can occur in tumors and be propagated in PDX models [32], amplification in breast tumors may be a relatively infrequent event and, thus, not likely an attractive clinical target.

ESR1 Mutations in Primary Breast Tumors: a Controversy Resolved

The general consensus for almost 20 years was that *ESR1* mutations in primary disease were either not present or were very rare [41]. Recently, TCGA reported no *ESR1* mutations in 390 primary tumors, apparently confirming this consensus opinion [33]. However, in 2000, our group reported a somatic K303R *ESR1* mutation in one third of premalignant breast hyperplasias and, subsequently, found it in almost 50% of primary breast tumors [42, 43]. K303R *ESR1* mutation studies are listed in Table 1. Consistent with TCGA data, several earlier reports failed to detect the K303R *ESR1* mutation in primary tumors using standard fluorescent sequencing technologies [47, 48]. We subsequently reported that the discrepancy was due to poor base incorporation in both the forward and reverse strands of *ESR1* using dye-labeled terminator sequencing techniques employed by these earlier investigators [43]. Conway et al. have also reported this problem with detection of the *ESR1* K303R mutation in invasive breast tumors, albeit the reported frequency of the mutation was low (6%) in their studies [44]. It has also recently been shown that the K303R mutation frequency (29%) was higher in tumors from women with a family history of breast cancer [46] and that mutation-positive cases were more likely to have a first-degree family history of breast cancer [45]. We assert that it is time to challenge the long-standing dogma concerning *ESR1* mutations in primary cancer, and conduct a reevaluation of the contradictory data that exists in the literature, rather than dismiss these data as outliers [49]. It is especially important to reconsider sequence detection methods, as the K303R mutation cannot be resolved with traditional core sequencing or NGS. Preliminary data using a sensitive droplet digital polymerase chain reaction (ddPCR) method confirm that the K303R mutation is indeed present at a low frequency, but often resides in a small subpopulation within primary tumors (Gu and Fuqua, unpublished).

Overexpression of the K303R *ESR1* mutation in ER-positive breast cancer cells conferred hypersensitivity to estrogen-stimulated growth [42], consistent with the hypersensitive growth of long-term estrogen-deprived MCF-7 breast cancer cells. K303R-expressing cells also displayed reduced sensitivity to Tam treatment, but only when growth factor signaling was engaged [50], demonstrating that enhanced growth factor receptor-ER crosstalk is one mechanism

Table 1 Summary of relevant K303R-positive patient cohorts

Study	Frequency	Method	Location detected	Reference
Fuqua et al. 2000	34% (20/59)	PCR amplification	Typical hyperplasia	[42]
Herynk et al. 2007	50% (133/267)	SNaPshot™	Invasive breast cancer	[43]
Conway et al. 2004, 2006	5.7% (37/653)	SSCP	Invasive breast cancer	[44, 45]
Abbasi et al. 2013	10.7% (16/150)	SSCP	Invasive breast cancer	[46]

of relative endocrine resistance associated with expression of this *ESR1* mutation. Expression of the K303R mutation also resulted in significantly increased hormone-independent activity and conferred resistance to AIs through dynamic interactions with the IGF-1R signaling pathway [51–53]. The K303R mutation altered genomic transcriptional output with enhanced expression of components both upstream and downstream of the IGF-1R receptor signaling network [54]. This mutation is a classical gain-of-function mutation with the lysine to arginine substitution rendering the receptor an enhanced substrate for phosphorylation by several kinase cascades [53, 55]. Finally, the mutation enhanced bidirectional communication with and response to signals from the microenvironment [56], an important, though underexplored, area of ER crosstalk. The influence of *ESR1* mutations on paracrine signaling is an exciting potential area for mutation-specific therapeutic intervention.

The K303R mutation was associated with recurrence-free survival in univariate analyses of tumors from 267 untreated breast cancer patients, but it was not an independent prognostic factor for outcomes in multivariate analyses [43]. Its presence was also associated with biologic measures of poor outcome, including larger tumor size, older age, and axillary lymph node positivity. Since the mutation is present in untreated patients, and not the consequence of treatment selection in primary tumors, it will be important to determine whether the mutation occurs spontaneously, is driven by exogenous carcinogens or hormone exposure, or is the result of endogenous DNA damage. We originally proposed that the K303R *ESR1* mutation may confer a proliferative advantage in premalignant lesions due to its hypersensitivity, especially in postmenopausal women with lower levels of circulating estrogen. Similarly, cells with this hyperproliferative mutation could provide a favorable environment to facilitate the accumulation of additional mutational events that drive tumor progression. Studies examining this mutation in metastatic tumors are underway.

"Rediscovery" of *ESR1* Mutations in the HBD

A number of years ago, we originally hypothesized that maintenance of ER expression, along with selection of specific *ESR1* mutations, was a key event in breast cancer progression, most likely due to the selective pressure of hormonal treatment in metastatic patients [7]. Our argument was based not only on identifying two key *ESR1* mutational hot spots [42, 57] but also on the fact that these mutations could provide biologic functions that would be selected for in emerging populations of metastatic deposits, e.g., estrogen hypersensitivity and/or independence.

In 1997, 30 tumors from MBC patients were screened for *ESR1* mutations using single-strand polymorphism conformation analyses coupled with Sanger sequencing techniques [57]. Sequencing was performed from polymerase chain reaction (PCR)-amplified DNA primed with *ESR1*-specific oligonucleotide primers, similar to the reduced exome sequencing performed in the contemporary laboratory setting. Three missense mutations were detected (S47T, K531E, and Y537N), corresponding to a 10% mutation frequency rate in metastatic tumors. The frequency of specific HBD *ESR1* mutations detected in individual sequencing studies is shown in Table 2. S47T and K531E were found to exhibit transcriptional activity similar to WT ER, and were not studied further. The Y537N mutation displayed potent, estradiol-independent activity in ER-negative breast cancer cells but was virtually unaffected by estradiol, Tam, or fulvestrant treatment. At that time, it was speculated that if present in other metastatic tumors, the mutation might contribute to breast cancer progression, and its constitutive activity might present as endocrine-resistant disease. It is important to note that the patient with the Y537N substitution presented with advanced stage IV breast cancer, and it was an ER, progesterone receptor (PR)-negative metastatic bone tumor that was sequenced after treatment with diethylstilbestrol (DES) therapy. Since estrogen treatment had little effect on the high constitutive activity of the Y537N mutation, and the patient sample was clinically ER, PR-negative, it is unlikely that a mutant subclonal population was selected for by DES treatment. Unfortunately, after this single report, sensitive *ESR1* gene-specific PCR amplification and sequencing of metastatic breast tumors was not validated by other laboratories, and surprisingly, the therapeutic implications of *ESR1* mutations in breast tumors were underappreciated until now.

Sixteen years later, two laboratories published correlating results using NGS of metastatic tumors, validating the presence of estrogen-independent *ESR1* mutations occurring within the HBD of ER [59, 60]. Both reports found highly recurrent mutations surrounding the site of the original mutation detected in 1997 (L536S, Y537S, D538G), demonstrating that this location is most likely a genomic "hot spot" for activating HBD *ESR1* mutations. Robinson et al. [59] sequenced 11 metastatic patients and showed that 54% harbored *ESR1* mutations. Toy et al. [60] sequenced two cohorts of patients and reported frequencies of 25–50 and 11% for HBD *ESR1* mutations, respectively. In a retrospective study of 217 ER-positive patients, Niu et al. used NGS to determine a 12.1% frequency of HBD *ESR1* mutations [64]. All specimens in this study were collected from MBC treated with at least one line of AIs, most of which were heavily pretreated with other hormonal agents. Most patients also carried three or more additional genomic alterations. Three patients achieved stable disease with a combination of exemestane and everolimus, though notably, all of these patients harbored only the D538G mutation. Sufficiently powered clinical studies are necessary to determine whether discrete mutations may respond differently to targeted therapies.

Table 2 Summary of reported frequencies of ligand-binding domain mutations

Study	Amino acid change	Frequency	Source
Zhang et al. 1997 [57]	Y537N	3.3% (1/30)	Metastasis
Li et al. 2013 [32]	Y537S	13.6% (3/22)	Metastasis
	E380Q	4.5% (1/22)	Metastasis
Merenbakh-Lamin et al. 2013 [58]	D538G	38% (5/13)	Metastasis
Robinson et al. 2013 [59]	L536Q	9% (1/11)	Metastasis
	Y537S	27% (3/11)	Metastasis
	D538G	18% (2/11)	Metastasis
Toy et al. 2013 [60]	Y537S	14% (5/36)	Metastasis
	D538G	8% (3/36)	Metastasis
	S463P	3% (1/36)	Metastasis
	L536R	3% (1/36)	Metastasis
	V534E	3% (1/36)	Metastasis
	Y537N	3% (1/36)	Metastasis
De Mattos-Arruda et al. 2014 [61]	E380Q	2% (7/287)	Primary tumor
	E380Q	68% (106/157)	Metastasis
	E380Q	46% (339/737)	CTC
	E380Q	19% (158/823)	CTC
	E380Q	58% (160/275)	CTC
	E380Q	53% (534/1009)	CTC
Jeselsohn et al. 2014 [62]	E380Q	0.74% (1/134)	Metastasis
	Y537N	1.15% (2/134)	Metastasis
	Y537S	1.15% (2/134)	Metastasis
	Y537C	1.15% (2/134)	Metastasis
	D538G	1.72% (3/134)	Metastasis
	344insC	1.15% (2/134)	Metastasis
Guttery et al. 2015 [63]	E380Q	2% (1/48)	cfDNA
	Y537S	2% (1/48)	cfDNA
	D538G	2% (1/48)	cfDNA
Niu et al. 2015 [64]	Y537S	5% (11/217)	Metastasis
	D538G	4% (9/217)	Metastasis
	Y537C	2% (4/217)	Metastasis
	Y537N	1% (2/217)	Metastasis
	V533 M	0.5% (1/217)	Metastasis
	L536P	0.5% (1/217)	Metastasis
	Y537P	0.5% (1/217)	Metastasis
	D538P	0.5% (1/217)	Metastasis
Sefrioui et al. 2015 [65]	Y537S	29% (2/7)	Metastasis
	D538G	43% (3/7)	Metastasis
	Y537N	29% (2/7)	Metastasis
Schiavon et al. 2015 [66]	D538G	11% (15/128)	cfDNA
	Y537S	2% (3/128)	cfDNA
	Y537N	3% (4/128)	cfDNA
	Y537C	2% (2/128)	cfDNA
	L536R	2% (2/128)	cfDNA
Takeshita et al. 2015 [67]	Y537S	45% (5/11)	Metastasis
	Y537C	27% (3/11)	Metastasis
	Y537N	36% (4/11)	Metastasis
	D538G	36% (4/11)	Metastasis

Table 2 (continued)

Study	Amino acid change	Frequency	Source
Wang et al. 2015 [68]	D538G	13% (2/15)	Metastasis
	Y537C	7% (1/15)	Metastasis
Chandarlapaty et al. 2016 [69]	Y537S	13.3%	cfDNA
		7.8% (72/541)	
	D538G	21.1% (114/541)	cfDNA
Clatot et al. 2016 [70]	Y537S	14.6% (21/144)	cfDNA
	Y537N	11.1% (16/144)	cfDNA
	Y537C	1.4% (2/144)	cfDNA
	D538G	16.7% (24/144)	cfDNA
Gyanchandani et al. 2016 [71]	D538G	18.8% (3/16)	cfDNA
	Y537S	12.5% (2/16)	cfDNA
	Y537N	12.5% (2/16)	cfDNA
Hrebien et al. 2016 [72]	D538G	21.1% (15/71)	cfDNA
Ma et al. 2016 [73]	E380Q	6.3% (1/16)	Primary tumor/metastasis
	S576L	6.3% (1/16)	Primary tumor/metastasis
Spoerke et al. 2016 [74]	Y537N	4% (2/47) (of mets)	Metastasis
	Y537N	4% (2/47)	cfDNA
	L536Q	2% (1/47)	Metastasis
	L536Q	2% (1/47)	cfDNA
	Y537C	6% (3/47)	Metastasis
	Y537C	6% (3/47)	cfDNA
	D538G	9% (4/47)	Metastasis
	D538G	11% (5/47)	cfDNA
	P535H	2% (1/47)	Metastasis
	P535H	2% (1/47)	cfDNA
	E380Q	13% (6/47)	Metastasis
	E380Q	15% (7/47)	cfDNA
	Y537S	2% (1/47)	Metastasis
	Y537S	2% (1/47)	cfDNA
	L536H	2% (1/47)	cfDNA
	S463P	2% (1/47)	cfDNA
Takeshita et al. 2016 [75]	Y537S	14% (6/42)	cfDNA
	D538G	5% (2/42)	cfDNA
	Y537N	12% (5/42)	cfDNA
Wang et al. 2016 [76]	Y537C	3% (1/29)	cfDNA
	Y537S	4% (1/24)	Metastasis
	Y537S	7% (2/29)	cfDNA
	D538G	7% (3/43)	Primary
	D538G	11.4% (4/35)	Metastasis
	D538G	21% (6/29)	cfDNA
Chu et al. 2017 [77]	Y537S	26.3% (5/19)	Metastasis
	D538G	52.6% (10/19)	Metastasis
	Y537N	10.5% (2/19)	Metastasis
Fribbens et al. 2017 [78]	D538G	14.2% (51/360)	cfDNA
	Y537N	3.9% (14/360)	cfDNA
	Y537S	6.4% (23/360)	cfDNA
	E380Q	6.1% (22/360)	cfDNA
	S463P	1.1% (4/360)	cfDNA
	Y537C	1.4% (5/360)	cfDNA
	L536R	0.3% (1/360)	cfDNA

Table 2 (continued)

Study	Amino acid change	Frequency	Source
Page et al. 2017 [79]	Y537N	3% (1/39)	cfDNA
	Y537S	5% (2/39)	cfDNA
	E380Q	8% (3/39)	cfDNA
Shaw et al. 2017 [80][a]	E380Q	50% (2/4)	CTC
	Y537C	25% (1/4)	CTC
	D538G	25% (1/4)	CTC
Yanagawa et al. 2017 [1]	Y537C	2% (1/46)	Metastasis
	Y537N	2% (1/46)	Metastasis
	D538G	2% (1/46)	Metastasis
	Y537S	5%(2/38)	cfDNA
	G557R	2% (1/46)	Metastasis
	D538G	8% (3/38)	cfDNA
	S463P	3% (1/36)	cfDNA
	L536H	3% (1/36)	cfDNA

[a] Study looked at 112 women with MBC, only 5 were analyzed for CTCs, 4 being ER+

Newer studies posit that *ESR1* mutations are indeed present in primary tumors, but only occur at very low frequencies [81]. *ESR1* mutations have been reported in low frequencies in primary tumors: detected at < 1% in a recent analysis of 772 primary TCGA tumors [82] and reported at 2.4% in TCGA primary tumors. *ESR1* mutations have also been detected using NGS in one primary ovarian cancer tumor [83] and four cases of endometrial cancer in the TCGA database [59]. Thus, understanding *ESR1* mutational effects could have implications beyond just breast cancer. These studies also demonstrate that *ESR1* mutations are detectable in primary tumors; however, due to their low frequencies, NGS may not be adequately sensitive for diagnostic purposes.

The more sensitive ddPCR can detect rare point mutations in small tumor subpopulations. Our lab identified *ESR1* mutations in primary breast tumors at a frequency of 2–12% using ddPCR [81]. Sequencing using ddPCR has also been used to estimate *ESR1* mutation frequencies in metastatic samples. The BOLERO-2 trial [69] enrolled women to evaluate the efficacy of adding the mTOR inhibitor everolimus to the steroidal AI exemestane to treat advanced MBC that had progressed on nonsteroidal AIs. In a retrospective study utilizing cell-free DNA (cfDNA) from 541 evaluable patients, ddPCR detected *ESR1* mutations in 28.8% of patients. Importantly, compared to patients with WT *ESR1*, these mutations were associated with shorter overall survival. Additionally, while both the D538G and WT patients' progression-free survival increased with the addition of everolimus, Y537S mutation-bearing patients received no added benefit with combined therapy. While this difference may be due to the lack of statistical power, it could also indicate that the HBD mutations promote different biological phenotypes. In 2015, Takeshita et al. [67] also applied ddPCR technology to a study of 55 metastatic breast cancer samples. They found a comparable overall frequency of 20% of the most frequent *ESR1* HBD mutations (Y537N, Y537S, D538G, and Y537C). They also noted a 2.5% frequency of mutations in primary breast tumors. They reported that half of the *ESR1* mutations found in MBC were not present in the corresponding primary tumor, and a few patients harbored double or triple mutations. These studies confirm that *ESR1* mutations are readily detectable using ddPCR and occur in both primary and MBC and that the mutational profile can be polyclonal.

From these studies, comprehensively listed in Table 2, it is clear that *ESR1* mutations occur at low frequencies in primary breast tumors, but at higher frequencies in MBC. These studies also indicate that *ESR1* mutations can be associated with shorter progression-free survival, and may need to be treated based on precise mutation type. However, in order to treat *ESR1* mutations specifically and effectively, the mechanisms by which *ESR1* mutations impact cancer progression must first be unraveled.

Biologic Mechanisms Associated with *ESR1* Mutations

Fewer than 20% of MBC tumors lose ER expression, and ER initiates invasion and promotes metastasis [7]. In light of recently published studies demonstrating the high frequency of *ESR1* mutations in MBC, we must understand how mutant ERs promote metastasis. Early studies on the K303R mutation revealed altered ER phosphorylation status [51]. This increased ER phosphorylation at several residues promotes interaction with growth factor signaling and enhanced binding of coactivators [51]. It is not known if a similar mechanism

may present in the hot spot HBD mutations. Studies to date have focused on understanding the conformational changes created by mutations in the HBD.

The Y537 residue is important for ER dimerization and activation in response to ligand binding [84], which is required for ER to bind DNA and initiate gene transcription [54]. Structural analyses have suggested that both the Y537S and D538G mutations lead to a novel hydrogen bond with the mutated amino acid [58, 60, 85]. This changes the receptor to an agonist conformation even in the absence of ligand, resulting in increased coactivator recruitment, decreased recruitment of Hsp90 chaperone protein, and in one study, increased phosphorylation at the S118 ER residue [60]. MCF-7 cells transfected with mutant ER showed increased hormone-independent transcriptional activation, proliferation, xenograft growth in vivo, and a decreased response to selective estrogen receptor downregulators (SERDs) [58, 60, 85]. Of note, one of these studies observed an additional hydrogen bond in the backbone of the D538G mutated receptor and an apparently smaller overall conformational change than that seen in the Y537S mutation [60]. As the BOLERO-2 study noted different responses to treatment between these two mutations [69], this structural alteration could be therapeutically important. However, in order to fully understand how specific individual mutations affect patient response, it will be essential to detect and monitor them clinically.

Monitoring *ESR1* Mutations In Vivo: a Moving Target

To answer the imperative question of how to best treat patients with *ESR1* mutations, a reliable method to sequentially monitor the evolution and emergence of mutants during treatment and tumor progression must be established. Since *ESR1* mutations are rarely detected in primary tumors, we must be able to ascertain when they appear during tumor progression, and monitor their emergence during treatment and metastasis to assess how to change therapeutic strategies.

Circulating tumor cells (CTCs) can be used to monitor changes in the genomic landscape of tumors during disease progression. Yu et al. [86] isolated CTCs from patient's plasma and created stable in vitro cultures where they then characterize a number of *ESR1* mutations. Though no *ESR1* mutations were observed in primary tumors, they were detected in metastatic samples and, interestingly, in tumor cells grown under low estrogen conditions in vitro. CTCs from patients with active metastatic disease who had ER-positive primary tumors retained ER expression. The authors also tested in vitro response to a number of single and combination targeted therapies. They found that mutant-harboring CTC lines were relatively resistant to selective estrogen receptor modulators (SERMs), SERDs, and the mTOR inhibitor everolimus, but were uniquely sensitive to Hsp90 inhibition. Though it is sometimes difficult to isolate enough CTCs from patients to perform sequencing, this study demonstrates that CTCs can be cultured in vitro to test therapeutic sensitivity.

Isolating cfDNA from the plasma of breast cancer patients is an attractive alternative to isolating CTCs. Several groups have compared cfDNA to CTC DNA and have found that cfDNA faithfully recaptures the genomic landscape of both CTCs and the original tumor [61, 80]. Despite great intra- and intertumoral heterogeneity, *ESR1* mutations are generally enriched or appear in serial biopsies in patients progressing over time, with little to no mutations detected in the primary tumor. However, when the mutation was present in the primary tumor, it was maintained in the metastasis [85].

As summarized in a recent review [87], utilizing serial plasma cfDNA samples as "liquid biopsies" to monitor *ESR1* mutation progression is an exciting diagnostic option. Liquid biopsies can be obtained at primary diagnosis and through the course of treatment and progression to MBC, where tissue biopsies are often difficult or impossible to obtain. Subsequent liquid biopsies postsurgery, postadjuvant treatment, and during metastatic progression could then be analyzed by ddPCR for the emergence and enrichment of *ESR1* mutations. This clinical scenario allows for the detection of *ESR1* mutant subclones during their emergence in disease progression, different lines of treatment, and metastatic onset. This clinical paradigm will likely elucidate how *ESR1* mutations' impact tumor response to therapy and, importantly, determine how *ESR1* mutations impact tumor evolution.

Evolution of *ESR1* Mutations During Treatment Course

ESR1 mutant cells could either evolve during tumor progression due to the selective pressure of treatment or represent an initial small subset of cells that evade treatment, as represented in Fig. 1. Indeed, compared to other molecular alterations, *ESR1* mutations appear to be a bona fide acquired mutation with the high discordant frequency between primary and metastatic tumors [62, 88]. In Fumagalli et al., *ESR1* mutations were found in 10.8% of patients who had received at least 5 years of hormone therapy. They noted that of the many alternations (p35, PI3K, etc.) identified, only *ESR1* exhibited discordance between primary and metastatic samples, indicating a unique role for *ESR1* mutations in promoting tumor progression.

It has long been appreciated that there is clonal heterogeneity within tumors. Miller et al. sought to define how this clonal heterogeneity could be remodeled by sequencing 22 primary tumors before and after neoadjuvant AI therapy [89]. They found that in three cases, AI therapy selected for the emergence and enrichment of *ESR1* mutations. This small

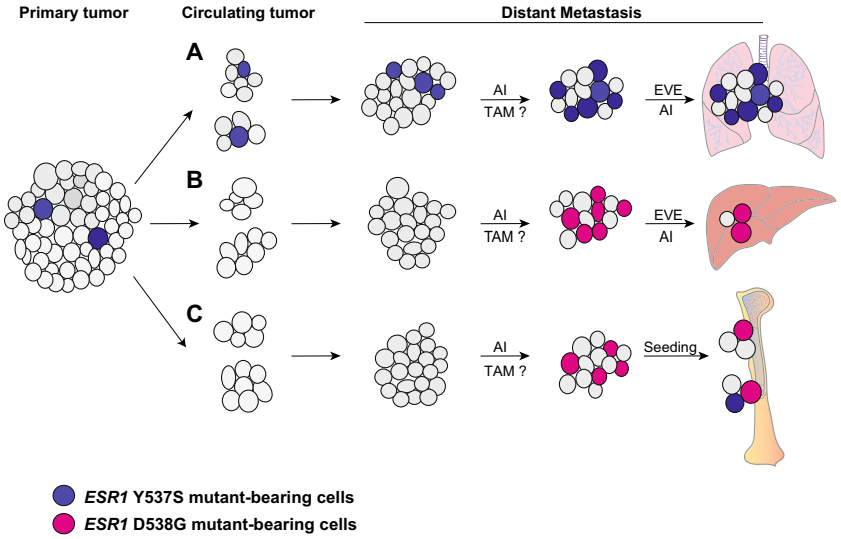

Fig. 1 Observed patterns of clonal evolution in mutant-bearing tumors. A primary tumor contains few to no *ESR1* mutant-bearing cells. However, it can seed clusters of circulating cells that eventually become mutant-expressing metastases through several different evolutionary patterns. As demonstrated in panel A, circulating cell clusters may bear the same mutation, here Y537S, as the primary tumor. When the circulating cell clusters colonize a distal organ and become a metastatic tumor, they may continue to express the mutation, even increasing in frequency. Treatment with an AI (and possibly a SERD such as Tam, though clinical evidence is lacking) in the metastatic setting may actually cause an increase in mutant frequency. As seen in the BOLERO-2 trial [69], combinatorial treatment in AI + Eve was not effective in Y537S-bearing tumors. In B, the primary tumor seeds circulating cell clusters which lack *ESR1* mutations, even in the metastatic tumor. However, hormonal treatment may provide selective pressure for the emergence of *ESR1* tumors in the metastatic setting. In this case the mutation is D538G, which does respond to AI + Eve treatment. In C, hormonal therapy again selects for expression of the D538G mutation, and the process of seeding for micrometastases selects for emergence of a second mutation.

study underscores the concept that therapy can influence clonal evolution, and enrich for *ESR1* mutations. More studies with greater power are needed to address the outstanding question: does the choice of endocrine agent influence clonal expansion of *ESR1* mutations?

Although a proof-of-principle study using cfDNA to predict relapse in early breast cancer patients failed to note *ESR1* mutations as having significant predictive value [90], another study successfully used cfDNA to track *ESR1* mutant evolution [66]. They noted an enrichment in *ESR1* clones only in MBC patients treated with AI exclusively in the metastatic setting. This evolutionary pattern was also observed in the BOLERO-2 trial [69] and in a preclinical study of *ESR1* mutations in PDX tumors or in vitro cell lines [32]. This observation could have implications for the treatment of MBC patients, both those who present with *ESR1* mutations in the primary setting, and to prevent the expansion of *ESR1* mutations in the metastatic setting. These studies are likely the "tip of the iceberg," as noted in Gu and Fuqua [87], and further underscore the importance of therapeutic monitoring.

ESR1 Mutations' Response to Treatment

Preclinical studies have predicted that tumors bearing *ESR1* HBD mutations will exhibit different responses to treatment compared to those with WT ER. In a retrospective study using ddPCR sequencing of cfDNA from patients with MBC, Takeshita et al. noted that the frequency of ER mutations changes over time and with treatment [75]. They observed that *ESR1* mutations increased over time and with AI therapy, supporting the hypothesis that these clones may be adapting to AI therapeutic conditions. The presence of *ESR1* mutations was correlated with shorter time to treatment failure; 83% of MBC patients with *ESR1* mutations exhibited a poor response to and shorter duration of effective endocrine control.

The BOLERO-2 trial [69, 91] saw an important change in *ESR1* mutation prevalence with respect to the timing of AI therapy. A threefold increase in *ESR1* mutation frequency was seen in patients treated with an AI for the first time in the metastatic setting (33 vs. 11%), compared to those who were treated with an AI in the adjuvant setting. This confirmed what an earlier smaller study [90] had observed: mutations were rarely seen in patients after adjuvant AI, implying that treatment was enriching *ESR1* mutations in the metastatic setting [66]. A similar observation was also made in the phase II FERGI trial, which enrolled women with MBC who had failed previous AI therapy. The FERGI trial added a pan-PI3K inhibitor to fulvestrant treatment and concluded that tumors bearing *ESR1* mutations were rarely found before AI treatment but were prevalent in MBC that had progressed on AI therapy [74].

These critical studies highlight four points: (1) the need for mutation tracking through cfDNA in plasma, (2) the importance of the timing of AI treatment in the adjuvant vs. metastatic setting, and (3) the possibility that AI treatment alone in the metastatic

setting may provide a positive selection for hormone-independent *ESR1* mutations, although it has recently been reported that Tam-only-treated MBC can harbor mutations [92], and (4) the potential importance of fulvestrant for the effective treatment of MBC. It is of paramount importance to unravel these questions, ideally through long-term follow-up of patients with *ESR1* mutations. These studies will likely reinforce the current standard of care for AI adjuvant therapy after primary detection but might indicate against the current practice of treating MBC patients with steroidal AI therapy alone. It also predicts the importance of alternate antiestrogen (fulvestrant and novel targeted agents) therapies in the treatment of MBC with *ESR1* mutations.

SERM/SERDs in the Treatment of Tumors with Mutant *ESR1*

If AI treatment provides a selection pressure for mutant-bearing clonal outgrowth in patients, the next option for therapy is likely a more effective SERM and/or SERD. Various studies have predicted that *ESR1* mutations will still respond to SERMs or SERDs, though perhaps at a decreased sensitivity based on in vitro, xenograft, and PDX preclinical models [32, 58, 60, 85]. A study using MCF-7 cells with CRISPR-Cas9 knock-in of the Y537S *ESR1* mutation found that cells were partially resistant to Tam and fulvestrant [93]. Tam has never been shown to be more effective with higher dosing [94]; however, fulvestrant may [95, 96]. Fulvestrant at 500 mg is more effective in preventing progression in ER mutant-bearing tumors [95]. This indicates that high-dose fulvestrant may be a promising therapeutic option. It also highlights the need for the development of novel, more potent SERMs/SERDs.

The quest for better SERDs has recently taken into consideration how these novel SERDs might specifically address the clinical consequences of *ESR1* mutations. Oral SERDs are an especially attractive alternative to fulvestrant, whose dose is limited due to the amount of drug feasible to inject intramuscularly. The oral SERD GDC-0810 was effective in in vitro and xenograft models. The authors specifically included an elegant CRISPR-Cas9 knock-in model of mutant ER Y537S [97] to test GDC-0810's ability to inhibit mutant cell growth. GDC-0810 was able to competitively bind mutant ER. A phase I clinical trial to evaluate this promising drug is currently recruiting patients (NCT01823835). A second new oral SERD, termed AZD9496, significantly inhibited PDX growth expressing the D538G mutation [98]. In an inducible mutant-expressing cell line, AZD9496 was also able to downregulate mutant ER and block PR induction better than Tam or fulvestrant. AZD9496 is currently in a phase I trial (NCT02248090). Finally, combining an oral SERD with targeted therapy (PI3K pathway inhibitor or CDK4/6 inhibitor) showed increased inhibitory effects compared to monotherapy alone [98], underscoring the potential utility of combinatorial therapy in mutant patient populations.

Combination Therapy to Treat Tumors with *ESR1* Mutations

An effective strategy to treat MBC patients with *ESR1* mutations is to combine selected targeted therapy with hormone therapy. With studies showing *ESR1* mutants' decreased sensitivity to SERDs and SERMs, the need for alternative targets for therapy is immediate. Harrod et al. combined fasudil with a CDK7 inhibitor in an MCF-7 Y537S knock-in preclinical model [93]. This combination reversed initial resistance to single agent therapy, promoted complete growth suppression, and reduced phosphorylation of ER at S118. Using RNA-Seq, they found that mutant ER had a unique gene set compared to WT ER, including elevated CDK7 expression. They hypothesized that mutant-bearing tumors might be more sensitive to targeted therapy aimed at suppressing the activity of these super-induced genes. CDK7 inhibitors have not yet been tested in MBC.

The PALOMA-1 clinical trial combined the AI letrozole with the CDK4/6 inhibitor palbociclib and found a significant increase in progression-free survival with the combined treatment compared to letrozole alone. In a smaller study of 16 patients with *ESR1* mutation-positive MCB treated with letrozole plus palbociclib, no difference in progression-free survival or overall survival was observed compared to WT tumors [71]. Unfortunately, this treatment regimen did not prevent an enrichment of *ESR1* mutations during treatment. Wardell et al. utilized a novel SERM-SERD hybrid (SSH) in combination with palbociclib in in vitro and animal PDX models of *ESR1*-expressing resistant cells [99]. They found that both a SERD-palbociclib and an SSH-palbociclib combination effectively inhibited growth in these preclinical models. Clinical testing of novel SERM/SERD/SSHs in combination with palbociclib is currently underway (single dose SERD GDC-9010 plus palbociclib phase II NCT01823835; SSH bazedoxifene plus palbociclib phase II/I NCT02448771). Palbociclib is also being evaluated clinically in combination with hormonal therapy (letrazole or tamoxifen plus palbociclib phase II NCT03065621; tamoxifen plus palbociclib as first line metastatic therapy phase II NCT02668666; fulvestrant plus palbociclib as second line therapy after progression on AI plus palbociclib phase II NCT02738866).

Conclusions

Though the frequency and relevance of *ESR1* mutations in breast cancer has been debated for nearly 20 years, recent

studies clearly demonstrate that *ESR1* mutations are present in primary and metastatic tumors, are enriched in the metastatic setting, and affect progression-free survival and response to hormone therapy. The frequency of *ESR1* HBD mutations in primary tumors remains low and sometimes undetectable with traditional sequencing techniques. In MBC patients, *ESR1* HBD frequencies range from 12 to 40%. Though some sequencing methods are very sensitive for detection of subclonal *ESR1* mutations, the ddPCR technology of plasma DNA is efficient for detection of *ESR1* mutations even at low frequencies. Thus, *ESR1* mutation status can be monitored real-time throughout treatment and disease progression. Mutations are a bona fide, and probably the dominant, mechanism of acquired hormone resistance in patients; their influence on response to therapy thus warrants deep exploration.

If ongoing clinical trials confirm what preliminary studies have observed, *ESR1* HBD mutations may warrant new clinical approaches to the management of MBC. These mutations appear to be enriched during treatment with an AI alone in MBC. This observation leads to three clinical possibilities which should be studied: (1) adjuvant treatment of primary tumors could include Tam or fulvestrant treatment sequencing to prevent enrichment of *ESR1* mutations, (2) continual monitoring of *ESR1* mutations during AI monotherapy, and (3) MBC patients bearing *ESR1* mutations should not be treated with a steroidal AI alone. The unmet clinical need for better SERM/SERD/SSHs is clear, and more potent SERDs and SSHs are currently entering clinical trials. Another option for treating *ESR1* mutations currently in clinical trials is the combination of hormonal therapy with targeted therapy, most promisingly fulvestrant plus palbociclib.

The long duration of recurrence risk in women with ER-positive breast cancer is recognized, and how best to precisely determine who should receive prolonged endocrine therapy would be of great value. The apparent heterogeneity of ER-positive breast cancer and the mechanisms of resistance dictate a paradigm shift toward translational research using liquid biopsy specimens from patients that progress during endocrine therapy in the metastatic setting. Though there is still much to be discovered about the role of *ESR1* mutations in breast cancer, there is enough evidence to conclude that the detection of *ESR1* mutations should now be considered an ancillary diagnostic test in patients with disease progression during AI treatment. Our continued understanding of the mechanisms behind *ESR1* mutations' effects on tumor progression and metastasis will be imperative to best arm women for the fight against their breast cancer.

Tam tamoxifen, *AIs* aromatase inhibitors, *ER* estrogen receptor, *ESO-ESMO* European School of Oncology-European School of Medical Oncology, *HER2* human epidermal growth factor receptor 2, *ESR1* estrogen receptor gene, *WT* wild-type, *NGS* next generation sequencing, *PDX* patient-derived xenograft, *HBD* hormone-binding domain, *TCGA* The Cancer Genome Atlas, *FISH* fluorescent in situ hybridization, *ddPCR* droplet digital polymerase chain reaction, *PR* progesterone receptor, *DES* diethylstilbestrol, *cfDNA* cell-free deoxyribonucleic acid, *MBC* metastatic breast cancer, *CTCs* circulating tumor cells, *SERM* selective estrogen receptor modulator, *SERD* selective estrogen receptor degrader, *SSH* SERM/SERD hybrid, *IC50* half maximal inhibitory concentration

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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