Published in final edited form as:
*Breast Cancer Res Treat*. 2014 February ; 144(1): 11–19. doi:10.1007/s10549-014-2847-4.

# Estrogen Receptor (ER) Mutations in Breast Cancer: Hidden in Plain Sight

**Suzanne AW Fuqua**, **Guowei Gu**, and **Yassine Rechoum**
Lester and Sue Smith Breast Center, Baylor College of Medicine, Dan L Duncan Cancer Center, Houston, TX 77030

## Abstract

The idea that somatic ER mutations could play an important role in the evolution of hormone dependent breast cancers was proposed some years ago [1,2], but has remained controversial until recently. A significant amount of new data has confirmed these initial observations and shown their significance, along with much additional work relevant to the treatment of breast cancer. Thus, it is the purpose of this review to summarize the research to date on the existence and clinical consequences of ER mutations in primary and metastatic breast cancer.

### Keywords

estrogen receptor; metastasis; tumor evolution; mutation; resistance

## Introduction

We will revisit the hypothesis put forth about the role of ER mutations in breast cancer [1], and scrutinize recent data along with their clinical implications. It was hypothesized that maintenance of ER expression, along with the selection of specific ER mutations, were key events in breast cancer progression, most probably due to the selective pressure of hormonal treatment (Fig. 1, reprinted from [1]), and current research is moving quickly in support of this original hypothesis. The majority of breast cancers express ER, where it functions as a ligand-dependent transcription factor driving tumor growth and survival. We know that in the absence of hormone, histone deacetylase and receptor co-repressors are bound to the receptor, and silence transcription. When hormone binds to the receptor, the activated receptor complex displaces repressor proteins and acetyltransferases are recruited to the complex, along with co-activator proteins such as the p160 co-activator (SRC 1-3) complex, thereby initiating transcription [2]. Thus ER's ligand-dependence can be viewed as Nature's “brake” on the receptor— “no gas, no go”.

All endocrine therapies in breast cancer target the ER signaling pathway, either by directly antagonizing ER , with (e.g. fulvestrant; Faslodex™) or without (tamoxifen [Tam]; Nolvadex™) enhancing its degradation, or by strategies that deprive the receptor of estrogen

Address for correspondence: Suzanne AW Fuqua, PhD, Lester and Sue Smith Breast Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, Fax: 713-798-1673, sfuqua@bcm.edu.

(aromatase inhibitors [AIs]). However, these treatments generate different cellular stresses, like the generation of reactive oxygen species with Tam treatment, and the creation of an estrogen-deprived but estrogen-hypersensitive environment with AI treatment [3,4]. These stresses could affect residual circulating tumor cells, occult metastatic tumor deposits, and ER itself. Thus different mechanisms evolve to evade these hormonal treatments [3,5,6]. We know that adjuvant endocrine therapy can halve the recurrence rate of patients with ER-positive breast cancer, but that late recurrences continue to occur many years after initial diagnosis. The majority of metastatic recurrences arising in ER-positive patients also retain ER expression, signifying a selection process where maintenance of ER expression must be important for tumor progression. Approximately 30-40% of patients with ER-positive metastatic disease will also respond to first-line hormonal therapies, and another 20% will experience disease stabilization, thus responses to hormonal therapy can last for many years in some patients with ER-positive breast cancer. Unfortunately, the median survival of patients with metastatic breast cancer is still only 2-3 years [7,8] with very few patients surviving 20 years after diagnosis of metastases [9]. We need to improve these outcomes, especially in metastatic disease, and we urgently need to block the emergence of late recurrences after adjuvant endocrine therapy.

## Breast Tumor Heterogeneity and Evolution

Recent comprehensive genomic studies demonstrated the extreme genetic heterogeneity of primary breast tumors, with the vast majority of mutations occurring at very low frequencies [10]. Notable exceptions were the frequent mutation of the p53 tumor suppressor gene in basal ER-negative tumors, and PIK3CA mutations in luminal ER-positive primary tumors. A study comparing patient-derived xenografts (PDX) from a patient with a basal breast cancer suggested that primary tumors can undergo genomic evolution, and that minor sub-populations with metastatic potential pre-exist and can emerge during the metastatic process [11]. Deep genomic sequencing of breast tumors has afforded an even clearer picture of the life history of breast tumors, identifying both gene drivers and frequent subclonal expansions during tumor progression [12]. Single cell sequencing has recently confirmed that primary tumors are composed of subclonal populations, where single cell clonal expansion can often seed metastasis [13]. The implication of this pioneering work is that metastases can occur late during tumor development from an advanced primary tumor expansion. Important remaining questions are how does treatment affect this process, and can we target those genes which confer a selective metastatic growth advantage? Answers may lie in the evolution of ER mutations in some patients which confer relative resistance to hormonal therapy.

## Resistance Due to Mutation of the Clinical Target

Precision in targeted therapy involves first the identification of key pathways which drive a patient's tumor growth, the complete blockade of these pathways, but also anticipation of escape pathways (e.g. resistance mechanisms) so that they can also be blocked therapeutically. Thus combination hormonal and biologic therapy approaches are required for optimal targeted therapy of ER-positive patients. This strategy was proven feasible with the successful use of the mTOR signaling pathway inhibitor everolimus, combined with a

steroidal AI, in breast cancer patients with advanced ER-positive breast cancer previously treated with nonsteroidal AIs [14]. Also, patients with HER2-positive tumors may not always require chemotherapy, but instead more complete blockade of HER receptor family members which dimerize with HER2, plus combined blockade of ER will be an effective and rational combination strategy [15].

Mutation of the clinical target is a common resistance mechanism in tumors. For instance, during the past few years, a genetically defined reclassification of non small cell lung cancer (NSCLC) has emerged depending on the specific mutations present. Although not very common in frequency, two subsets are defined by EGFR or ALK activating mutations which confer high sensitivity and durable responses to tyrosine kinase inhibitors [16]. Another subset of NSCLC patients contain BRAF mutations which provide mechanisms of resistance to therapy [17]. Thus, tumor-specific mutations may act in at least two ways. In certain genes, activating mutations might confer enhanced sensitivity, perhaps through “addiction” to a specific growth or survival pathway [18]. Alternatively, mutations can confer resistance to targeted therapy, for instance, the specific BRAF mutations in NSCLC described above. Similarly, mutations in PI3KCA in ER-positive breast cancer have been reported to be associated with better responses to tamoxifen monotherapy [19], but predict poor response to other targeted therapies such as dual-HER2-targeted therapy [20,15]. Although infrequent, mutations in the HER2 oncogene are also now being reported in breast tumors [21], and studies exploring their role in resistance to HER2-targeted therapies are underway.

Thus, it is a surprising fact that the presence of ER mutations in breast tumors has been largely overlooked during the genomic reclassification of breast tumors in the past decade. Mutation of the clinical target, in this case ER , is finally an emerging and accepted certainty, as will be detailed below. It is unfortunate that the paucity of reports of ER mutations in tumors led many to assume that they were not there, an assumption that was based on the analysis of relatively few breast tumor samples with standard, less-sensitive sequencing technologies [22].

## The Hypersensitive K303R ER mutation—An Actionable Target

ER is modified by many post-translational modifications (PTMs), such as phosphorylation, sumoylation, acetylation, methylation, and ubiquination [23]. ER PTMs can affect its transcriptional activity, its protein turnover, co-activator and co-repressor binding, and response to hormone-mediated cellular signaling [24,25]. A lysine 303 to arginine (K303R) somatic ER mutation was reported in 2000, occurring in a third of premalignant breast hyperplasias, and one half of invasive breast tumors [26]. This mutation resides at the boundary between the so-called “hinge region” of the receptor and its hormone binding domain. The hinge region contains sites for many regulatory controls, such as binding of co-activators and co-repressors, and PTMs. The presence of the K303R ER mutation was controversial for a number of years with several laboratories failing to detect the mutation in invasive tumors using dye-labeled terminator core sequencing. However, Conway et al. subsequently reported in 2005 that the mutation was indeed present in primary breast tumors in a population-based study using an alternative screening strategy, though the frequency of detection with their method was low (5.7%) [27]. Of course, in hindsight with the overall

low gene mutation frequency in primary tumors reported by the Cancer Genome Atlas (TCGA) group of investigators, even this lower frequency of detection was significant because most breast cancer mutations are reported to occur in less than 1-5% of cases [28,10]. In 2007 the detection controversy was resolved, showing that the failure to detect the K303R ER mutation via dye-labeled terminator sequencing was due to the specific 3 and 4-base pair combinations preceding the mutation in both the forward and reverse strands [29]. It has been shown that specific base combinations can affect base pair height reads at the 3′ base. For instance, the 3-bp combination GAA results in a reduced peak height for the 3′ base; thus automated base calling will only detect wild-type sequence at this base, and this 3-base combination is the same sequence combination in the K303R ER mutation [29]. The K303R ER mutation can be reliably detected with methods such as SnapShot™, which uses primer extension sequencing across the mutation site (Fig. 2). Similarly, Dr. Conway's group showed that methods such as single strand conformation polymorphism (SSCP), which relies on base composition and size for electrophoretic separation of mutant alleles, was effective at resolving the presence of the mutation in invasive tumors [30]. SSCP technology was also used back in 1996 to detect variant RNA splice forms of the receptor [31], which now are being detected with RNA sequencing (RNA Seq) technologies. The inability of high-throughput sequencing methods, such as the Illumina™ sequencing technology, to detect the K303R ER mutation is thus consistent with its not being reported within the large, publically available TCGA database of primary breast tumors. Undoubtedly there are a small percentage of tumor-specific mutations which are not resolved with Next Generation Sequencing.

There is much known about the molecular mechanisms of altered activities associated with the K303R ER mutation, and the clinical implications of these alterations. These studies show that ER mutations are actionable clinical targets. The K303R ER mutation alters response to the antiestrogen Tam, but only in a cellular background with elevated cellular growth factor stimulation which converts Tam into an agonist *in vitro* in mutation-expressing cells. The lysine to arginine mutation creates a new AKT phosphorylation site at ER serine (S) 305 [32], which is an important phosphorylation site in modulating ER's response to Tam [33]. The mutation enhances constitutive phosphorylation at the S305 site as well, which has been shown to be important for several aspects of antiestrogen action [32]. The mutation blocks acetylation sites within this region preventing this PTM [34] that then affects other downstream PTM events, such as methylation, thereby stabilizing the receptor [35]. The K303R ER mutation also exhibits increased binding with members of the SRC co-activator family (SRC-1 and 2), and decreased binding to receptor co-repressors, such as NCOR1 and BRCA1, leading to increased promoter occupancy [26,36,37], which then enhances ligand-independent activity of the receptor. This ligand-independent activity could provide a selective growth advantage, especially in postmenopausal women with breast cancer where circulating levels of estrogen are low but still capable of stimulating the growth of mutant-expressing cells. As a consequence untreated primary tumors which express this mutation are associated with aggressive clinical features, such as larger tumor size and lymph node-positive status [29].

The K303R mutation also exhibits increased binding to growth factor receptors, such as HER2 and insulin-like growth factor receptor [IGF1R]), which further enhances downstream signaling molecules, such as the p85 regulatory subunit of PI3K [32,38,39], Activation of IGF1R in primary tumors has also been reported [28], highlighting that evolving breast tumors may use multiple, parallel or complementary mechanisms of resistance to survive (e.g. mutation of ER and activation of IGF1R) coordinately providing a selective growth advantage during treatment. Overexpression of the K303R mutation also confers hypersensitivity to estrogen growth stimulation [38,40], again a selective growth advantage compared to wild-type receptor in women with low circulating levels of estrogen. In preclinical models, cells expressing the mutation also exhibit enhanced communication with cytokines, such as leptin, leading to increased invasive growth [41]. Importantly, the mutant receptor directs enhanced bidirectional cross-talk between ER and growth factor receptors such as HER2 and IGF1R [32,39], which again together could coordinately play an important role in resistance to hormonal agents.

AIs are the most effective hormonal treatment of ER -positive breast cancers, and are currently sequenced with Tam during the management of patients, with recent data demonstrating that extended durations of hormonal therapy may be needed for effective long-term control of late recurrence [42]. To explore the role of the K303R mutation and subsequent activation at ER S305 in AI resistance ($AI^R$), a preclinical model was developed via stable transfection of an aromatase expression vector in K303R-overexpressing breast cancer cells [43]. The aromatase substrate androstenedione (AD) increased growth via its conversion to estrogen by aromatase in this model, and the AI anastrozole (Ana) decreased AD-stimulated growth of wild-type ER -expressing cells, whereas mutant-expressing cells were resistant to the inhibitory effect of Ana on growth. Resistance occurred through constitutive activation of the PI3K/Akt pro-survival signaling pathway to which the mutant cells had become addicted for maintenance of growth [38], and specific IGFR1R and Akt inhibitors restored AI sensitivity in mutant-expressing cells. The clinical implications of this translational research are that mutant-expressing cells may escape from growth inhibition when treated with AIs, and that the development of treatment strategies utilizing specific biologic inhibitors may be required to extend the duration of sensitivity to estrogen deprivation, or to reverse resistance at its emergence. Thus the growth addiction of the K303R ER mutation might also prove to be its clinical “Achilles heel.”

Importantly, inhibiting S305 phosphorylation with a blocking peptide also inhibited IGF1R/IRS-1/Akt signaling, and restored AI sensitivity. It was hypothesized that the K303R mutation and the S305 ER residue are determinants of AI response, and that blockade of S305 phosphorylation represents a new therapeutic strategy for treating tumors resistant to hormone therapy. In summary, extensive preclinical data suggest that the K303R ER mutation might be a new predictive marker to identify patients who will develop resistance to hormonal therapy. We encourage investigators to examine primary and metastatic tumor samples using primer-extension sequencing methods (such as the SnapShot™ and Sequenom™ technologies) capable of detecting this important ER mutation site, so that its full clinical significance can be realized.

## Mutational Hot Spot in ER : Leucine (L) 536, Tyrosine (Y) 537, and Aspartate (D) 538

The Y537 to asparagine (N) mutation was discovered in 1997 occurring in 1/30 metastatic breast tumors [44]. This mutation eliminates a tyrosine residue that is an important c-Src phosphorylation site with roles in regulating ligand binding, dimerization, and transcriptional activity of ER [45,46] and replaces it with a residue which induced a conformational change which mimics hormone binding. The Y537N ER mutation exhibited highly elevated ligand-independent, constitutive transcriptional activity; thus cells expressing the mutant were resistant to tamoxifen treatment, and were only partially inhibited by treatment with the pure steroidal antiestrogen fulvestrant. It was postulated at that time that a mutation at this site induced a conformational change in ER allowing escape from normal phosphorylation-mediated regulatory controls, providing cells with a selective oncogenic advantage during tumor progression, especially during treatment with hormonal therapies [44]. Similarly, Dr. Benita Katzenellenbogen's group examining *in vitro*-derived mutations surrounding the Y537 site hypothesized that these mutations mimic many of the changes in ER properties which are normally under estrogen control [45]. Subsequently Dr. Murphy and colleagues reported that phosphorylation at Y537 ER was associated with poor clinical outcome in breast cancer patients treated with tamoxifen [47]. A recent study has demonstrated that ER phosphorylation at Y537 by Src can trigger promoter occupancy and specific target gene expression [48], thus this site may be a critical regulatory site.

Recently several reports have confirmed the earlier results on Y537, extending the number of different mutations at the Y537 ER site in metastatic breast tumors, and have demonstrated that this region corresponds to an ER mutational hot spot region with somatic mutations in the surrounding residues L536 and D538. Undoubtedly realization of these important results can be attributed to the power of deep Next Generation sequencing and genomic interrogation of metastatic tumors, an area which has been underexplored until recently. Dr. Ellis' group was the first to report that 3/15 metastatic tumor samples maintained as patient derived xenografts in mice harbored a Y537S mutation [49]. Like the Y537N mutation, the Y537S mutation exhibited high constitutive ER transcriptional activity and high levels of the estrogen-responsive progesterone receptor, and was only partially responsive to growth inhibition with fulvestrant treatment. These investigators also speculated that the mutation could arise as an adaption to endocrine therapy, potentially providing a mechanism of aromatase inhibitor resistance. Further strong support for the important role of this ER region comes from two reports just published [50,51]. In the study from Dr. Chinnaiyan's group, 5/11 metastatic tumors contained mutations in this region (L536G, Y537S, Y537C, Y537N, and D538G) [50]. In contrast to previous studies finding relative resistance to antiestrogens, these authors report that the ER transcriptional activity of all five mutations was inhibited by tamoxifen and fulvestrant. The reason for the differences between their results and others is not known; however growth response assays were not presented in this study. Mutations in this region appear to be frequent with 9/36 metastatic tumors sequenced by Toy et al. [51] harboring non-synonymous substitutions at this hotspot. Molecular dynamic simulations suggest that the structures of the Y537S and D538G mutants involve hydrogen bonding of the mutant favoring the agonist conformation

of the receptor [51]. This finding supports molecular structure-function work by earlier pioneering investigators [45,46]. Importantly Toy et al. report that hormone-independent growth and activity was observed in cells expressing mutations within this positional hotspot, and that mutant-expressing cell growth was only partially responsive to antiestrogens [51]. In summary, it is clear that mutations in this region might be particularly frequent in metastatic breast tumors, and warrant exploration for their utility as predictive markers in metastatic patients, especially those on aromatase inhibitor regimens.

Fig. 3 depicts the genomic location of all reported ER mutations to date in primary and metastatic breast tumors. The 303 and 536-538 ER receptor residues are undoubtedly two functionally-important sites for somatic gain-of-function mutations in primary and metastatic tumors, respectively. Although currently infrequent other mutations have been reported, for instance at the E380 site within the ligand binding domain [50], and the S118 residue which is a MAPK phosphorylation site that can significantly alter hormone binding and function [52-54]. The D538G mutation is significant in that it was identified in 5 liver metastases, but not in the matched primary tumors, from patients who had failed multiple hormonal therapies, [55]. Modeling of the D538G substitution leads to a conformational change in the ligand binding domain which mimics the conformation of activated ligand-bound receptor, and alters the binding of tamoxifen [55]. Many of the other additional mutations shown in Fig. 3 have previously been reviewed [56], and will not be detailed further herein. It is certainly easy to predict that further site-specific alterations in the ligand binding domain will be identified once deep genomic sequencing is routinely employed to interrogate metastatic lesions where tumor evolution and the selective pressures of treatment could encourage subclonal expansion during metastatic dissemination.

## Conclusions and Clinical Implications

Recent guidelines for the workup of metastatic breast cancer patients suggest that the first recurrence of disease should be biopsied [57], but this is not always done for a number of practical or conservative clinical reasons. General clinical acceptance of the high probability of ER mutations in tumors that will help inform treatment decisions should encourage the acquisition of biopsies from all metastatic patients when possible, especially those accessible tumors which grew while on hormonal therapies. Looking for ER mutations in metastatic tumors can be likened to the mystery in Poe's “Purloined Letter”—hidden in plain sight. It was predictable that such an important growth regulatory pathway like the ER transcriptional network in breast cancer would evolve and mutate to evade therapy. Thus as originally proposed [1], it is reasonable to assert that both the maintenance of ER expression, and the evolution of biological attributes which are advantageous for the complex process of invasion and metastasis, all which ER gain-of-function mutations can affect, would prove to be important for the progression of ER-positive breast tumors. These data also suggest that ER mutation analysis in metastatic tumors should become main-stream after appropriate prospective clinical studies confirming their utility, and that mutation analyses will then be used to make decisions on the hormonal treatment of patients with ER-positive recurrent breast cancers.

With the relative resistance of the reported mutations in metastatic disease to existing therapies, there is also an urgent need to search for better receptor antagonists. For instance, final results from the CONIRM trial demonstrated the therapeutic superiority of higher dose (500 mg) fulvestrant in postmenopausal women with locally advanced or metastatic ER-positive breast cancer that had recurred or progressed after prior hormone therapy [58]. It remains to be determined if tumors with specific ER mutations will be responsive to higher doses of fulvestrant.

Phosphorylation is a critically important regulator of receptor function. We need new biological approaches, such as stable peptide mimetics, to block the downstream effectors of mutant receptors. Such an approach was used to block the phosphorylation and cellular crosstalk of the K303R ER mutant receptor with the growth factor receptor pathway in preclinical models [32,39], and similar strategies could be employed for the other receptor mutational hotspots such as the Y537 site [59]. From a therapeutic standpoint, knowing the mutational status of the Y537 hotspot might only be helpful in women who present with metastatic disease where traditional hormonal blockade with tamoxifen or aromatase inhibitors might not be most effective. However, in cells where resistance occurs due to expression of the K303R ER mutation, hormone sensitivity can be restored by inhibitors which block mutant receptor crosstalk involving IGF1R and Akt signaling, suggesting that mutant-expressing cells may have become addicted to particular growth pathways. Thus, the K303R mutation is an actionable target with current therapeutic strategies. Determining whether the Y537 hotspot also has similar therapeutic vulnerability to biological targeted therapies should be a translational research priority.

Fig. 4 summarizes the potential clinical implications of ER mutation analysis in breast cancer. Primary tumors are treated with aromatase inhibitors, tamoxifen, or a serial combination. Both of these hormonal therapies are very effective in the adjuvant setting, with long durations of patient responses. Deep sequencing of primary tumors to detect subclonal populations expressing mutant receptors is probably clinically warranted to help appropriately guide adjuvant therapy. In addition, sequencing techniques which can detect the presence of the K303R ER mutation in primary tumors, even though its absolute frequency has not been established as of yet, are needed to help guide the treatment of patients with this mutation. Emerging technologies such as sequencing of circulating tumor cell (CTC) DNA might afford opportunities to monitor the progression of subclonal populations expressing relevant mutant receptors [60]. Upon first recurrence, genomic and proteomic analysis could identify both mutant cell populations and downstream effector pathways of mutant transcriptional activity, so that specific biologic targeting strategies could be offered along with switching of hormonal therapies to agents such as high dose fulvestrant. These proposed clinical approaches are feasible and could encompass a more rational precision paradigm for the future benefit of women with ER-positive breast cancer.

Finally, we must stress that ER mutations might also be important early during the evolution of premalignant lesions to primary cancer. The K303R ER mutation was originally discovered in premalignant atypical ductal hyperplasias with an even higher frequency detected in invasive breast tumors [26]. An ER mutation which confers a proliferative advantage, such as hypersensitivity to estrogen, could provide a favorable cellular

environment to accelerate the accumulation of additional genetic events important for tumor progression [26]. Indeed, Dr. Conway's group has also reported that the K303R ER mutation was a risk factor for breast cancer in women with a familial history of breast cancer [28]. The association of the K303R ER mutation with familial breast cancer risk has also been reported in Asian-Caucasian (Iranian) breast cancer patients; the frequency of the mutation in this cohort was 10% [30]. The use of deep sequencing techniques or single cell sequencing of DNA from heterogeneous lesions might afford a better understanding of not only the frequency of specific ER mutations, but also the life history and evolution of these mutations during breast tumor treatment and progression.

## Acknowledgments

I want to personally thank Dr. Jan Carlstedt-Duke for his continued encouragement and support of my ER mutational work in breast cancer. I also would like to acknowledge Ms. Amanda Beyer and Dr. Gary Chamness for excellent editorial assistance with this review. Finally, my sincere thanks go to Dr. Marc Lippman, who suggested the analogy to Poe's short story. This work was supported by NIH/NCI R01-CA72038.

## References

1. Fuqua SA. The role of estrogen receptors in breast cancer metastasis. J Mammary Gland Biol Neoplasia. 2001; 6(4):407–417. [PubMed: 12013530]
2. Dasgupta S, Lonard DM, O'Malley BW. Nuclear Receptor Coactivators: Master Regulators of Human Health and Disease. Annu Rev Med. 2013
3. Cui Y, Parra I, Zhang M, Hilsenbeck SG, Tsimelzon A, Furukawa T, Horii A, Zhang ZY, Nicholson RI, Fuqua SA. Elevated expression of mitogen-activated protein kinase phosphatase 3 in breast tumors: a mechanism of tamoxifen resistance. Cancer Res. 2006; 66(11):5950–5959. [PubMed: 16740736]
4. Santen RJ, Song RX, Zhang Z, Kumar R, Jeng MH, Masamura S, Lawrence J Jr, MacMahon LP, Yue W, Berstein L. Adaptive hypersensitivity to estrogen: mechanisms and clinical relevance to aromatase inhibitor therapy in breast cancer treatment. J Steroid Biochem Mol Biol. 2005; 95(1-5): 155–165. [PubMed: 16024245]
5. Razandi M, Pedram A, Jordan VC, Fuqua S, Levin ER. Tamoxifen regulates cell fate through mitochondrial estrogen receptor beta in breast cancer. Oncogene. 2013; 32(27):3274–3285. [PubMed: 22907432]
6. Schiff R, Reddy P, Ahotupa M, Coronado-Heinsohn E, Grim M, Hilsenbeck SG, Lawrence R, Deneke S, Herrera R, Chamness GC, Fuqua SA, Brown PH, Osborne CK. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. J Natl Cancer Inst. 2000; 92(23):1926–1934. [PubMed: 11106684]
7. Nicolini A, Giardino R, Carpi A, Ferrari P, Anselmi L, Colosimo S, Conte M, Fini M, Giavaresi G, Berti P, Miccoli P. Metastatic breast cancer: an updating. Biomed Pharmacother. 2006; 60(9):548–556. [PubMed: 16950593]
8. Smith TJ, Davidson NE, Schapira DV, Grunfeld E, Muss HB, Vogel VG 3rd, Somerfield MR. American Society of Clinical Oncology 1998 update of recommended breast cancer surveillance guidelines. J Clin Oncol. 1999; 17(3):1080–1082. [PubMed: 10071303]
9. Greenberg PA, Hortobagyi GN, Smith TL, Ziegler LD, Frye DK, Buzdar AU. Long-term follow-up of patients with complete remission following combination chemotherapy for metastatic breast cancer. J Clin Oncol. 1996; 14(8):2197–2205. [PubMed: 8708708]
10. Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, Nik-Zainal S, Martin S, Varela I, Bignell GR, Yates LR, Papaemmanuil E, Beare D, Butler A, Cheverton A, Gamble J, Hinton J, Jia M, Jayakumar A, Jones D, Latimer C, Lau KW, McLaren S, McBride DJ, Menzies A, Mudie L, Raine K, Rad R, Chapman MS, Teague J, Easton D, Langerod A, Oslo Breast Cancer C. Lee MT, Shen CY, Tee BT, Huimin BW, Broeks A, Vargas AC, Turashvili G, Martens J, Fatima A, Miron P, Chin SF, Thomas G, Boyault S, Mariani O, Lakhani SR, van de Vijver M, van

't Veer L, Foekens J, Desmedt C, Sotiriou C, Tutt A, Caldas C, Reis-Filho JS, Aparicio SA, Salomon AV, Borresen-Dale AL, Richardson AL, Campbell PJ, Futreal PA, Stratton MR. The landscape of cancer genes and mutational processes in breast cancer. Nature. 2012; 486(7403): 400–404. [PubMed: 22722201]

11. Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, Harris CC, McLellan MD, Fulton RS, Fulton LL, Abbott RM, Hoog J, Dooling DJ, Koboldt DC, Schmidt H, Kalicki J, Zhang Q, Chen L, Lin L, Wendl MC, McMichael JF, Magrini VJ, Cook L, McGrath SD, Vickery TL, Appelbaum E, Deschryver K, Davies S, Guintoli T, Lin L, Crowder R, Tao Y, Snider JE, Smith SM, Dukes AF, Sanderson GE, Pohl CS, Delehaunty KD, Fronick CC, Pape KA, Reed JS, Robinson JS, Hodges JS, Schierding W, Dees ND, Shen D, Locke DP, Wiechert ME, Eldred JM, Peck JB, Oberkfell BJ, Lolofie JT, Du F, Hawkins AE, O'Laughlin MD, Bernard KE, Cunningham M, Elliott G, Mason MD, Thompson DM Jr, Ivanovich JL, Goodfellow PJ, Perou CM, Weinstock GM, Aft R, Watson M, Ley TJ, Wilson RK, Mardis ER. Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature. 2010; 464(7291):999–1005. [PubMed: 20393555]
12. Nik-Zainal S, Van Loo P, Wedge DC, Alexandrov LB, Greenman CD, Lau KW, Raine K, Jones D, Marshall J, Ramakrishna M, Shlien A, Cooke SL, Hinton J, Menzies A, Stebbings LA, Leroy C, Jia M, Rance R, Mudie LJ, Gamble SJ, Stephens PJ, McLaren S, Tarpey PS, Papaemmanuil E, Davies HR, Varela I, McBride DJ, Bignell GR, Leung K, Butler AP, Teague JW, Martin S, Jonsson G, Mariani O, Boyault S, Miron P, Fatima A, Langerod A, Aparicio SA, Tutt A, Sieuwerts AM, Borg A, Thomas G, Salomon AV, Richardson AL, Borresen-Dale AL, Futreal PA, Stratton MR, Campbell PJ, Breast Cancer Working Group of the International Cancer Genome C. The life history of 21 breast cancers. Cell. 2012; 149(5):994–1007. [PubMed: 22608083]
13. Navin N, Kendall J, Troge J, Andrews P, Rodgers L, McIndoo J, Cook K, Stepansky A, Levy D, Esposito D, Muthuswamy L, Krasnitz A, McCombie WR, Hicks J, Wigler M. Tumour evolution inferred by single-cell sequencing. Nature. 2011; 472(7341):90–94. [PubMed: 21399628]
14. Baselga J, Campone M, Piccart M, Burris HA 3rd, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, Beck JT, Ito Y, Yardley D, Deleu I, Perez A, Bachelot T, Vittori L, Xu Z, Mukhopadhyay P, Lebwohl D, Hortobagyi GN. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2012; 366(6):520–529. [PubMed: 22149876]
15. Rimawi MF, Mayer IA, Forero A, Nanda R, Goetz MP, Rodriguez AA, Pavlick AC, Wang T, Hilsenbeck SG, Gutierrez C, Schiff R, Osborne CK, Chang JC. Multicenter phase II study of neoadjuvant lapatinib and trastuzumab with hormonal therapy and without chemotherapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer: TBCRC 006. J Clin Oncol. 2013; 31(14):1726–1731. [PubMed: 23569315]
16. Paolo M, Assunta S, Antonio R, Claudia SP, Anna BM, Clorinda S, Francesca C, Fortunato C, Cesare G. Selumetinib in advanced non small cell lung cancer (NSCLC) harbouring KRAS mutation: endless clinical challenge to KRAS-mutant NSCLC. Rev Recent Clin Trials. 2013; 8(2): 93–100. [PubMed: 24063423]
17. Gautschi O, Peters S, Zoete V, Aebersold-Keller F, Strobel K, Schwizer B, Hirschmann A, Michielin O, Diebold J. Lung adenocarcinoma with BRAF G469L mutation refractory to vemurafenib. Lung Cancer. 2013; 82(2):365–367. [PubMed: 24035431]
18. Weinstein B. Relevance of the concept of oncogene addiction to hormonal carcinogenesis and molecular targeting in cancer prevention and therapy. Adv Exp Med Biol. 2008; 617:3–13. [PubMed: 18497026]
19. Loi S, Haibe-Kains B, Majjaj S, Lallemand F, Durbecq V, Larsimont D, Gonzalez-Angulo AM, Pusztai L, Symmans WF, Bardelli A, Ellis P, Tutt AN, Gillett CE, Hennessy BT, Mills GB, Phillips WA, Piccart MJ, Speed TP, McArthur GA, Sotiriou C. PIK3CA mutations associated with gene signature of low mTORC1 signaling and better outcomes in estrogen receptor-positive breast cancer. Proc Natl Acad Sci U S A. 2010; 107(22):10208–10213. [PubMed: 20479250]
20. Rimawi MF. SABCS. abstract.
21. Loi S, Michiels S, Lambrechts D, Fumagalli D, Claes B, Kellokumpu-Lehtinen PL, Bono P, Kataja V, Piccart MJ, Joensuu H, Sotiriou C. Somatic mutation profiling and associations with prognosis and trastuzumab benefit in early breast cancer. J Natl Cancer Inst. 2013; 105(13):960–967. [PubMed: 23739063]

22. Roodi N, Bailey LR, Kao WY, Verrier CS, Yee CJ, Dupont WD, Parl FF. Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. J Natl Cancer Inst. 1995; 87(6):446–451. [PubMed: 7861463]
23. Barone I, Brusco L, Fuqua SA. Estrogen receptor mutations and changes in downstream gene expression and signaling. Clin Cancer Res. 2010; 16(10):2702–2708. [PubMed: 20427689]
24. Le Romancer M, Poulard C, Cohen P, Sentis S, Renoir JM, Corbo L. Cracking the estrogen receptor's posttranslational code in breast tumors. Endocr Rev. 2011; 32(5):597–622. [PubMed: 21680538]
25. Duplessis TT, Williams CC, Hill SM, Rowan BG. Phosphorylation of Estrogen Receptor alpha at serine 118 directs recruitment of promoter complexes and gene-specific transcription. Endocrinology. 2011; 152(6):2517–2526. [PubMed: 21505052]
26. Fuqua SA, Wiltschke C, Zhang QX, Borg A, Castles CG, Friedrichs WE, Hopp T, Hilsenbeck S, Mohsin S, O'Connell P, Allred DC. A hypersensitive estrogen receptor-alpha mutation in premalignant breast lesions. Cancer Res. 2000; 60(15):4026–4029. [PubMed: 10945602]
27. Conway K, Parrish E, Edmiston SN, Tolbert D, Tse CK, Geradts J, Livasy CA, Singh H, Newman B, Millikan RC. The estrogen receptor-alpha A908G (K303R) mutation occurs at a low frequency in invasive breast tumors: results from a population-based study. Breast Cancer Res. 2005; 7(6):R871–880. [PubMed: 16280033]
28. Conway K, Parrish E, Edmiston SN, Tolbert D, Tse CK, Moorman P, Newman B, Millikan RC. Risk factors for breast cancer characterized by the estrogen receptor alpha A908G (K303R) mutation. Breast Cancer Res. 2007; 9(3):R36. [PubMed: 17553133]
29. Herynk MH, Parra I, Cui Y, Beyer A, Wu MF, Hilsenbeck SG, Fuqua SA. Association between the estrogen receptor alpha A908G mutation and outcomes in invasive breast cancer. Clin Cancer Res. 2007; 13(11):3235–3243. [PubMed: 17545528]
30. Abbasi S, Rasouli M, Nouri M, Kalbasi S. Association of estrogen receptor-alpha A908G (K303R) mutation with breast cancer risk. Int J Clin Exp Med. 2013; 6(1):39–49. [PubMed: 23236557]
31. Zhang QX, Hilsenbeck SG, Fuqua SA, Borg A. Multiple splicing variants of the estrogen receptor are present in individual human breast tumors. J Steroid Biochem Mol Biol. 1996; 59(3-4):251–260. [PubMed: 9010317]
32. Barone I, Iacopetta D, Covington KR, Cui Y, Tsimelzon A, Beyer A, Ando S, Fuqua SA. Phosphorylation of the mutant K303R estrogen receptor alpha at serine 305 affects aromatase inhibitor sensitivity. Oncogene. 2010; 29(16):2404–2414. [PubMed: 20101208]
33. Michalides R, Griekspoor A, Balkenende A, Verwoerd D, Janssen L, Jalink K, Floore A, Velds A, van't Veer L, Neefjes J. Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. Cancer Cell. 2004; 5(6):597–605. [PubMed: 15193262]
34. Cui Y, Zhang M, Pestell R, Curran EM, Welshons WV, Fuqua SA. Phosphorylation of estrogen receptor alpha blocks its acetylation and regulates estrogen sensitivity. Cancer Res. 2004; 64(24): 9199–9208. [PubMed: 15604293]
35. Subramanian K, Jia D, Kapoor-Vazirani P, Powell DR, Collins RE, Sharma D, Peng J, Cheng X, Vertino PM. Regulation of estrogen receptor alpha by the SET7 lysine methyltransferase. Mol Cell. 2008; 30(3):336–347. [PubMed: 18471979]
36. Herynk MH, Hopp T, Cui Y, Niu A, Corona-Rodriguez A, Fuqua SA. A hypersensitive estrogen receptor alpha mutation that alters dynamic protein interactions. Breast Cancer Res Treat. 2010; 122(2):381–393. [PubMed: 19842032]
37. Ma Y, Fan S, Hu C, Meng Q, Fuqua SA, Pestell RG, Tomita YA, Rosen EM. BRCA1 regulates acetylation and ubiquitination of estrogen receptor-alpha. Mol Endocrinol. 2010; 24(1):76–90. [PubMed: 19887647]
38. Barone I, Cui Y, Herynk MH, Corona-Rodriguez A, Giordano C, Selever J, Beyer A, Ando S, Fuqua SA. Expression of the K303R estrogen receptor-alpha breast cancer mutation induces resistance to an aromatase inhibitor via addiction to the PI3K/Akt kinase pathway. Cancer Res. 2009; 69(11):4724–4732. [PubMed: 19487288]

39. Giordano C, Cui Y, Barone I, Ando S, Mancini MA, Berno V, Fuqua SA. Growth factor-induced resistance to tamoxifen is associated with a mutation of estrogen receptor alpha and its phosphorylation at serine 305. Breast Cancer Res Treat. 2010; 119(1):71–85. [PubMed: 19205871]
40. Herynk MH, Lewis MT, Hopp TA, Medina D, Corona-Rodriguez A, Cui Y, Beyer AR, Fuqua SA. Accelerated mammary maturation and differentiation, and delayed MMTVneu-induced tumorigenesis of K303R mutant ERalpha transgenic mice. Oncogene. 2009; 28(36):3177–3187. [PubMed: 19561644]
41. Barone I, Catalano S, Gelsomino L, Marsico S, Giordano C, Panza S, Bonofiglio D, Bossi G, Covington KR, Fuqua SA, Ando S. Leptin mediates tumor-stromal interactions that promote the invasive growth of breast cancer cells. Cancer Res. 2012; 72(6):1416–1427. [PubMed: 22282662]
42. Early Breast Cancer Trialists' Collaborative G. Peto R, Davies C, Godwin J, Gray R, Pan HC, Clarke M, Cutter D, Darby S, McGale P, Taylor C, Wang YC, Bergh J, Di Leo A, Albain K, Swain S, Piccart M, Pritchard K. Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. Lancet. 2012; 379(9814):432–444. [PubMed: 22152853]
43. Barone I, Cui Y, Herynk MH, Corona-Rodriguez A, Giordano C, Selever J, Beyer A, Ando S, Fuqua SA. Expression of the K303R estrogen receptor-alpha breast cancer mutation induces resistance to an aromatase inhibitor via addiction to the PI3K/Akt kinase pathway. Cancer research. 2009; 69(11):4724–4732. [PubMed: 19487288]
44. 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(7):1244–1249. [PubMed: 9102207]
45. Lazennec G, Ediger TR, Petz LN, Nardulli AM, Katzenellenbogen BS. Mechanistic aspects of estrogen receptor activation probed with constitutively active estrogen receptors: correlations with DNA and coregulator interactions and receptor conformational changes. Mol Endocrinol. 1997; 11(9):1375–1386. [PubMed: 9259327]
46. Zhong L, Skafar DF. Mutations of tyrosine 537 in the human estrogen receptor-alpha selectively alter the receptor's affinity for estradiol and the kinetics of the interaction. Biochemistry. 2002; 41(13):4209–4217. [PubMed: 11914066]
47. Skliris GP, Nugent Z, Watson PH, Murphy LC. Estrogen receptor alpha phosphorylated at tyrosine 537 is associated with poor clinical outcome in breast cancer patients treated with tamoxifen. Horm Cancer. 2010; 1(4):215–221. [PubMed: 21761367]
48. Sun J, Zhou W, Kaliappan K, Nawaz Z, Slingerland JM. ERalpha phosphorylation at Y537 by Src triggers E6-AP-ERalpha binding, ERalpha ubiquitylation, promoter occupancy, and target gene expression. Mol Endocrinol. 2012; 26(9):1567–1577. [PubMed: 22865929]
49. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, He X, Liu S, Hoog J, Lu C, Ding L, Griffith OL, Miller C, Larson D, Fulton RS, Harrison M, Mooney T, McMichael JF, Luo J, Tao Y, Goncalves R, Schlosberg C, Hiken JF, Saied L, Sanchez C, Giuntoli T, Bumb C, Cooper C, Kitchens RT, Lin A, Phommaly C, Davies SR, Zhang J, Kavuri MS, McEachern D, Dong YY, Ma C, Pluard T, Naughton M, Bose R, Suresh R, McDowell R, Michel L, Aft R, Gillanders W, DeSchryver K, Wilson RK, Wang S, Mills GB, Gonzalez-Angulo A, Edwards JR, Maher C, Perou CM, Mardis ER, Ellis MJ. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. Cell Rep. 2013; 4(6):1116–1130. [PubMed: 24055055]
50. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, Kalyana-Sundaram S, Wang R, Ning Y, Hodges L, Gursky A, Siddiqui J, Tomlins SA, Roychowdhury S, Pienta KJ, Kim SY, Roberts JS, Rae JM, Van Poznak CH, Hayes DF, Chugh R, Kunju LP, Talpaz M, Schott AF, Chinnaiyan AM. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013; 45(12):1446–1451. [PubMed: 24185510]
51. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, Li Z, Gala K, Fanning S, King TA, Hudis C, Chen D, Taran T, Hortobagyi G, Greene G, Berger M, Baselga J, Chandarlapaty S. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013; 45(12):1439–1445. [PubMed: 24185512]
52. Ali S, Metzger D, Bornert JM, Chambon P. Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J. 1993; 12(3): 1153–1160. [PubMed: 8458328]

53. Piccart M. AACR. 2012 abstract.
54. Sarwar N, Kim JS, Jiang J, Peston D, Sinnett HD, Madden P, Gee JM, Nicholson RI, Lykkesfeldt AE, Shousha S, Coombes RC, Ali S. Phosphorylation of ERalpha at serine 118 in primary breast cancer and in tamoxifen-resistant tumours is indicative of a complex role for ERalpha phosphorylation in breast cancer progression. Endocr Relat Cancer. 2006; 13(3):851–861. [PubMed: 16954434]
55. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, Yelensky R, Brown M, Miller VA, Sarid D, Rizel S, Klein B, Rubinek T, Wolf I. D538G Mutation in Estrogen Receptor-alpha: A Novel Mechanism for Acquired Endocrine Resistance in Breast Cancer. Cancer Res. 2013; 73(23):6856–6864. [PubMed: 24217577]
56. Herynk MH, Fuqua SA. Estrogen receptor mutations in human disease. Endocr Rev. 2004; 25(6): 869–898. [PubMed: 15583021]
57. Carlson RW, Allred DC, Anderson BO, Burstein HJ, Edge SB, Farrar WB, Forero A, Giordano SH, Goldstein LJ, Gradishar WJ, Hayes DF, Hudis CA, Isakoff SJ, Ljung BM, Mankoff DA, Marcom PK, Mayer IA, McCormick B, Pierce LJ, Reed EC, Smith ML, Soliman H, Somlo G, Theriault RL, Ward JH, Wolff AC, Zellars R, Kumar R, Shead DA, National Comprehensive Cancer N. Metastatic breast cancer, version 1.2012: featured updates to the NCCN guidelines. J Natl Compr Canc Netw. 2012; 10(7):821–829. [PubMed: 22773798]
58. Leo AD, Jerusalem G, Petruzelka L, Torres R, Bondarenko IN, Khasanov R, Verhoeven D, Pedrini JL, Smirnova I, Lichinitser MR, Pendergrass K, Malorni L, Garnett S, Rukazenkov Y, Martin M. Final Overall Survival: Fulvestrant 500mg vs 250mg in the Randomized CONFIRM Trial. J Natl Cancer Inst. 2013
59. Arnold SF, Notides AC. An antiestrogen: a phosphotyrosyl peptide that blocks dimerization of the human estrogen receptor. Proc Natl Acad Sci U S A. 1995; 92(16):7475–7479. [PubMed: 7543683]
60. Dawson SJ, Rosenfeld N, Caldas C. Circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013; 369(1):93–94. [PubMed: 23822788]
61. Anderson TI, Wooster R, Laake K, Collins N, Warren W, Skrede M, Elles R, Tveit KM, Johnston SR, Dowsett M, Olsen AO, Moller P, Stratton MR, Borresen-Dale AL. Screening for ESR mutations in breast and ovarian cancer patients. Hum Mutat. 1997; 9(6):531–536. [PubMed: 9195227]
62. cBio-portal. 2012
63. Garcia T, Sanchez M, Cox JL, Shaw PA, Ross JB, Lehrer S, Schachter B. Identification of a variant form of the human estrogen receptor with an amino acid replacement. Nucleic Acids Res. 1989; 17(20):8364. [PubMed: 2478962]
64. Giltane JM.
65. Jesselsohn. SABCS. 2013 abstract.
66. Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM. Estrogen receptor mutations in tamoxifen-resistant breast cancer. Cancer Res. 1994; 54(2):349–353. [PubMed: 8275466]

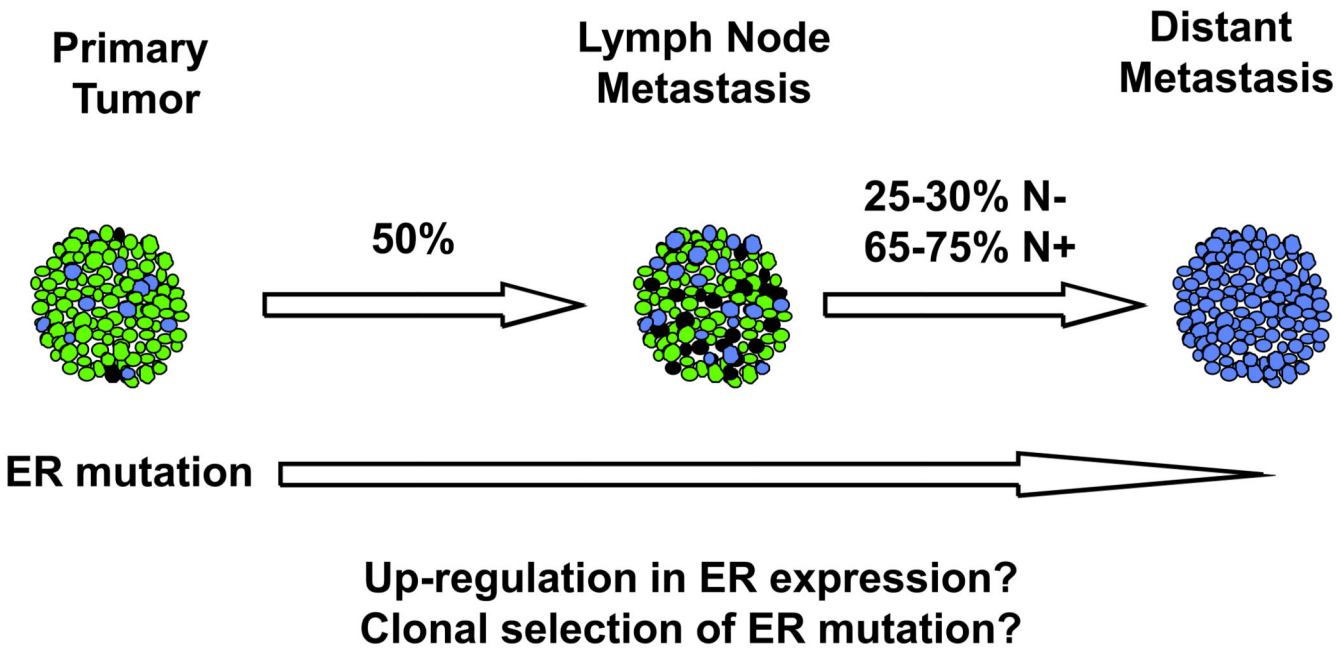


**Fig. 1.**

The progression of invasive breast cancer (IBC). Approximately 50% of primary breast tumors have already metastasized to the axillary lymph nodes upon presentation. Of these axillary node-positive (N+) patients, 65-75% will eventually develop distant metastasis, while only 25% of axillary node-negative (N-) patients will metastasize. We hypothesize that up-regulation of ER expression, and clonal selection of ER mutants are involved in the progression and metastasis of many breast tumors. Reprinted with kind permission from Springer Science+Business Media: Journal of Mammary Gland Biology and Neoplasia, The Role of Estrogen Receptors in Breast Cancer Metastasis, volume 6, 2001, page 409, Suzanne A.W. Fuqua, Figure 1.

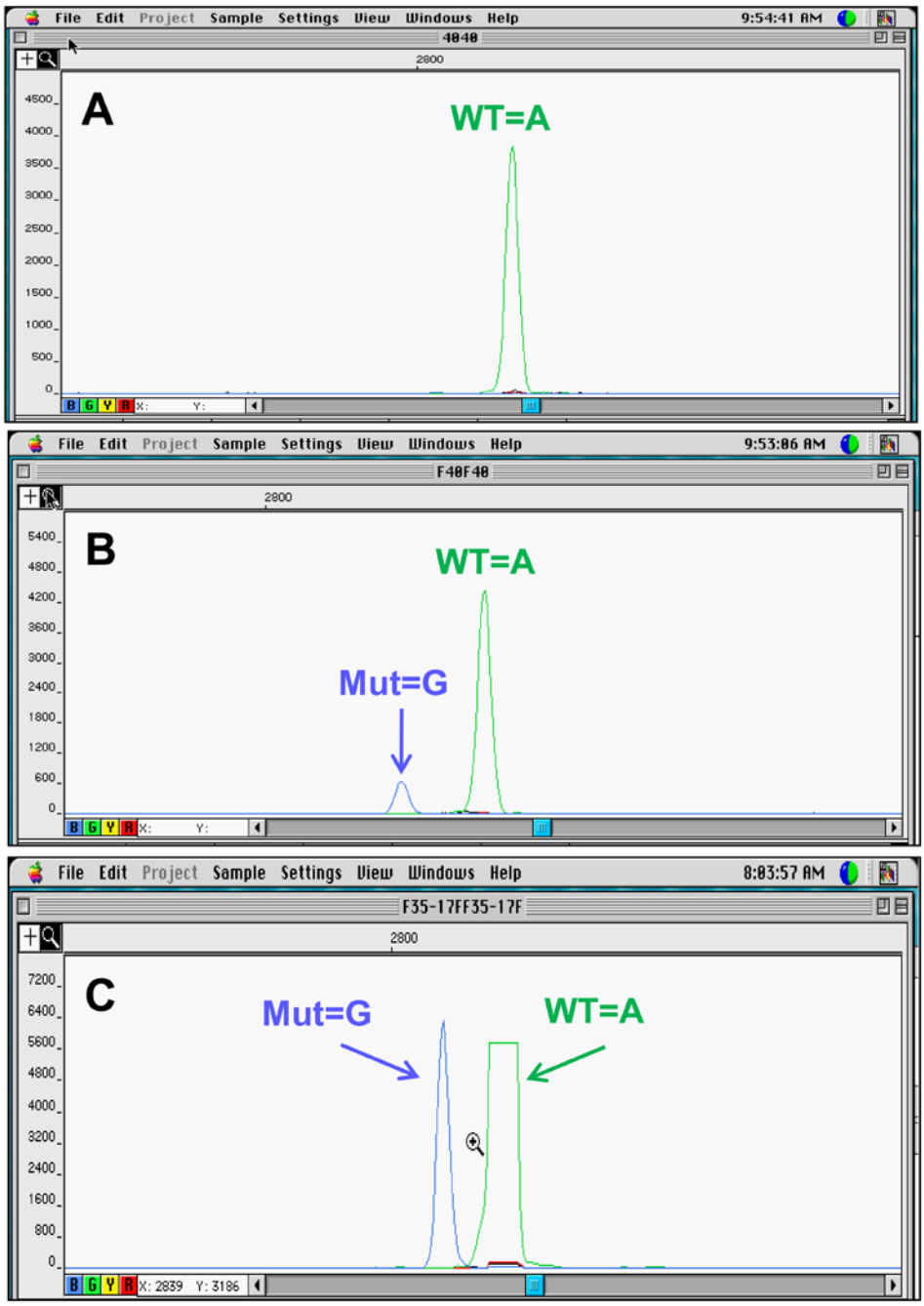


**Fig. 2.**

Detection of the K303R ER mutation requires optimized primer extension sequencing technology. Panel A shows primer extension sequencing (SnapShotTM) technology applied to high molecular weight DNA isolated from a frozen invasive breast tumor. Panel B shows the same DNA which had been sheared to approximately 200 bp which allows resolution of the mutation. Panel C shows enhanced detection of the mutation in sheared DNA from Panel B and the use of optimized primer size.

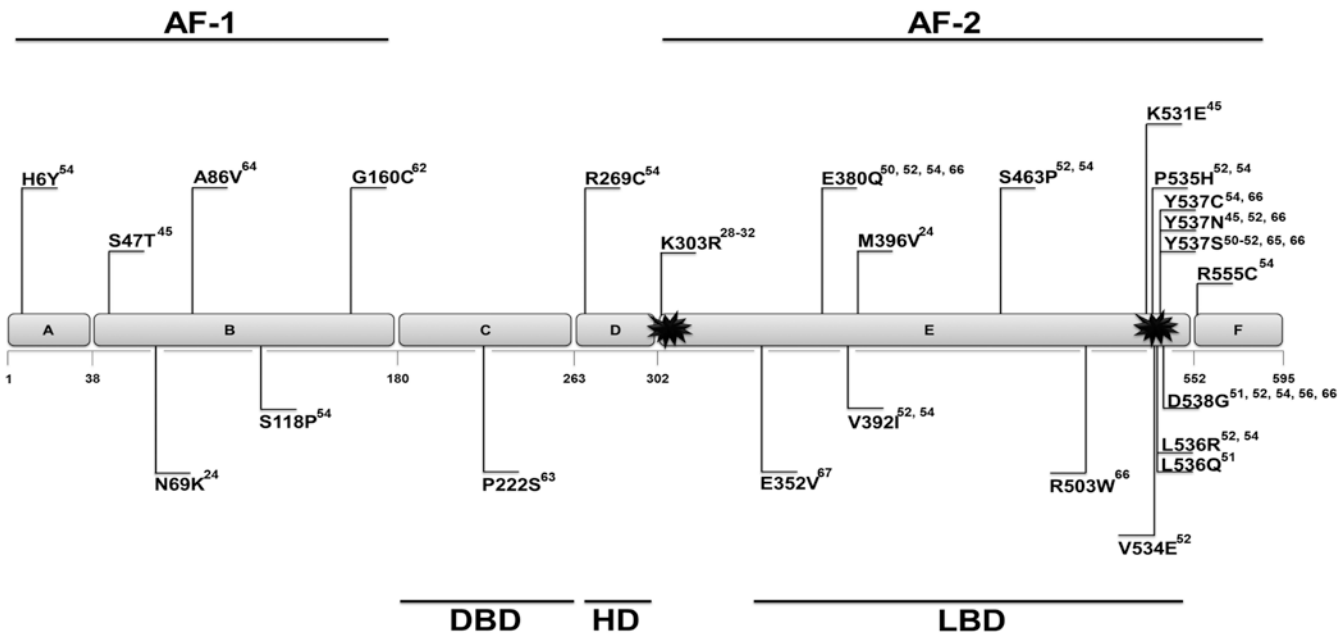


**Fig. 3.**

Current schemata of ER (ESR1 mutations) placed alongside their genomic locations and ER genomic structure. Hormone-independent activity is contained within the activation function 1 (AF-1) domain and hormone-dependent activity is contained within the activation function 2 (AF-2) domain. Mutations within AF-2 can cause hormone-independent activity. The hormone binding domain (HBD), hinge domain (HD), and ligand binding domain (LBD) locations are indicated. Amino acid resides are indicated. as well as functional A-E domains. References include [30,61,62,27,28,26,63,64,29,65,66,49,55,53,50,22,51,44].

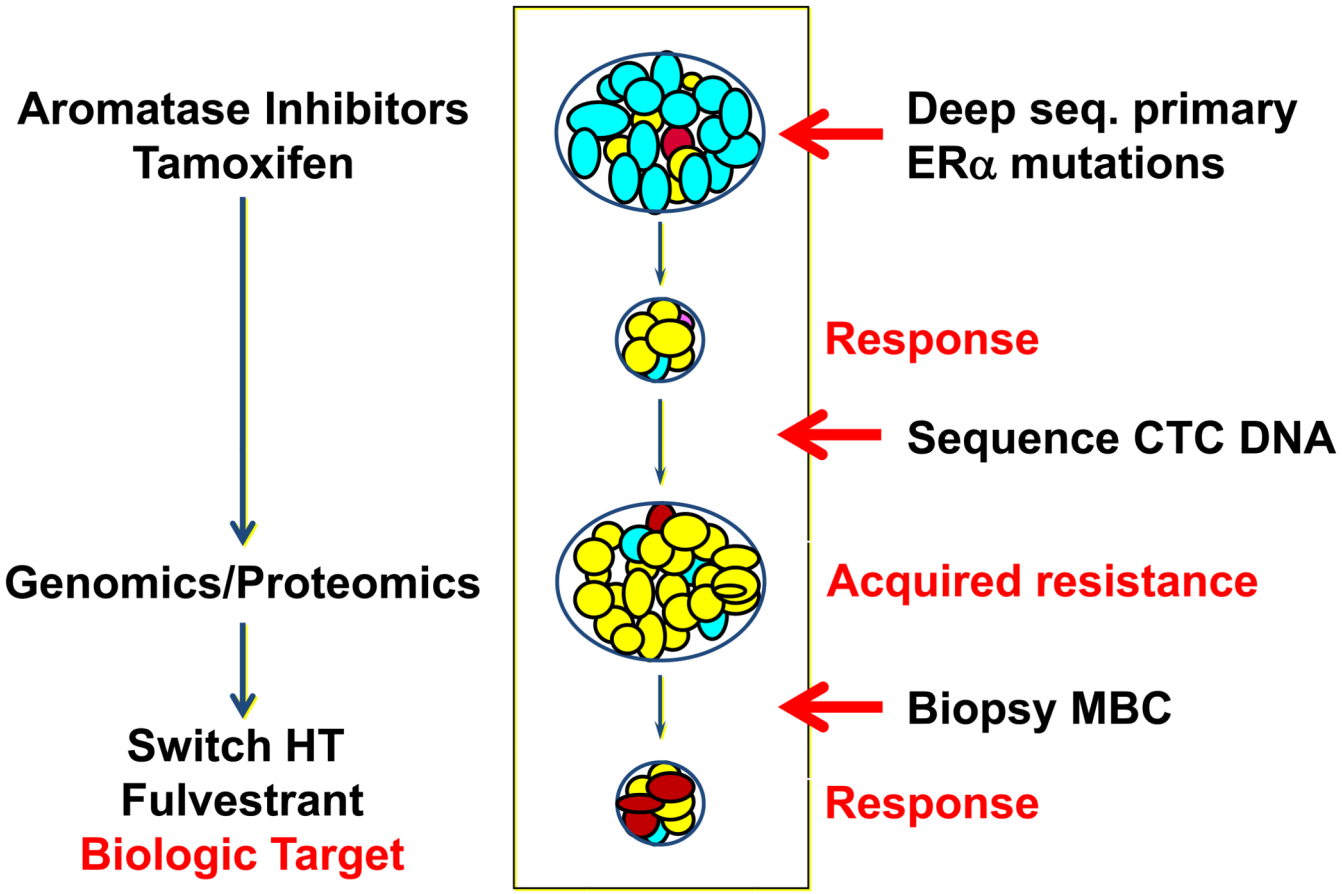


**Fig. 4.**

Clinical implications of ER mutations in both primary and metastatic breast cancers, and strategies to utilize mutations for clinical therapeutic decisions.