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ESR1 Mutations Affect Anti-proliferative Responses to Tamoxifen through Enhanced Cross-Talk with IGF Signaling

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Abstract

It is now generally accepted that estrogen receptor (ESR1) mutations occur frequently in metastatic breast cancers, however we do not yet know how to best treat these patients. We have modeled the three most frequent hormone binding ESR1 (HBD-ESR1) mutations (Y537N, Y537S, and D538G) using stable lentiviral transduction in human breast cancer cell lines. Effects on growth were examined in response to hormonal and targeted agents, and mutation-specific changes were studied using microarray and western blot analysis. We determined that the HBD-ESR1 mutations alter anti-proliferative effects to tamoxifen (Tam), due to cell-intrinsic changes in activation of the insulin-like growth factor receptor (IGF1R) signaling pathway and levels of PIK3R1/PIK3R3. The selective estrogen receptor degrader, fulvestrant, significantly reduced the anchorage-independent growth of ESR1 mutant-expressing cells, while combination treatments with the mTOR inhibitor everolimus, or an inhibitor blocking IGF1R and the insulin receptor significantly enhanced anti-proliferative responses. Using digital drop (dd) PCR we identified mutations at high frequencies ranging from 12% for Y537N, 5% for Y537S, and 2% for D538G in archived primary breast tumors from women treated with adjuvant mono-tamoxifen therapy. The HBD-ESR1 mutations were not associated with recurrence-free or overall survival in response in this patient cohort, and suggest that knowledge of other cell-intrinsic factors in combination with ESR1 mutation status will be needed determine anti-proliferative responses to Tam.

Keywords

Estrogen Receptor; Mutations; IGF-1; Fulvestrant; Everolimus

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Disclosure of Potential Conflicts of Interest

Authors have nothing to disclose

INTRODUCTION

Adjuvant hormonal therapy targeting the estrogen receptor (ESR1) in human breast cancer has significantly improved overall survival for patients whose tumors express this receptor¹. However, acquired resistance is a major clinical problem in breast cancer treatment. Several mechanisms of hormone resistance have been identified, including loss of estrogen receptor alpha (ER α) expression, altered activity of ER co-activators, and cross-talk with growth factor receptors such as HER2 and IGF1R². Recently it has been shown that mutations in the hormone binding domain (HBD) of ESR1 occur frequently in metastatic breast tumor tissues^{3,4,5,6,7}, and are selected for during aromatase inhibitor (AI) treatment⁸.

The first reported HBD-ESR1 mutation, a tyrosine to asparagine missense mutation at amino acid residue 537 (Y537N), was discovered in 1997 by cloning of cDNA from a metastatic lesion, and displayed strong constitutive hormone-independent transcriptional activity⁹. A second mutation at lysine 303 (K303R) was reported in both atypical ductal hyperplasias and invasive breast cancers, and was hypersensitive to low levels of estrogen^{10,11}. It was therefore proposed that somatic ESR1 mutations might be acquired during resistance to therapy, which could play important roles in the metastatic spread of breast cancer¹². However, this hypothesis remained controversial until a large number of metastatic tumors were interrogated using next generation sequencing^{3,4,5,6,7}. Many ESR1 mutations appear to occur at a mutational “hot spot” surrounding residues 536–538 of the HBD, shifting the receptor into a constitutive agonist conformation⁷ with stabilization of co-activator binding¹³. Investigators have consistently shown that these ESR1 mutations exhibit elevated estrogen-independent transcriptional activity^{3,4,5,6,7}. Recently it was suggested that the combination of constitutive recruitment of co-activators in the absence of hormone and reduced Tam binding affinity might underlie therapy resistance conferred by the Y537S and D538G ESR1 mutations¹³. HBD-ESR1 mutations are present at high frequencies in the plasma of metastatic patients using sensitive ddPCR technologies, and indeed may predict reduced sensitivity to subsequent aromatase AI therapy in the metastatic setting⁸.

The selective estrogen receptor degrader (SERD) fulvestrant is effective in ER-positive patients progressing on hormonal therapy, and is now considered part of a sequential treatment regimen for patients with metastatic disease¹⁴. FERGI trial investigators, who examined the addition of a phosphoinositide-3-kinase (PI3K) inhibitor with fulvestrant in AI-resistant metastatic patients, reported that the presence of HBD-ESR1 mutations did not predict resistance to fulvestrant¹⁵, thus fulvestrant combined with other targeted therapies may be a viable treatment option in patients with ESR1 mutations. The BOLERO-2 trial demonstrated a 50% reduction in progression when the mTOR inhibitor everolimus was combined with exemestane¹⁶, and a subsequent correlative analysis reported that patients with certain of the ESR1 mutations may benefit from this combination as well¹⁷.

Although the selective estrogen receptor modulator (SERM) Tam can block estrogen-stimulated ESR1 mutant transcriptional activity^{3,4,5,6,7}, it has not been shown whether Tam will block the growth of ESR1 mutant-expressing cells in patients. Herein, we report a preclinical study showing that sensitivity to the anti-proliferative effects of Tam in some ER-

positive cells expressing the HBD hotspot ESR1 mutations is dependent on cross-talk with the IGF1R signaling pathway.

RESULTS

HBD-ESR1 mutations modulate anti-proliferative responses to Tam depending on cellular background

We established stable pools of ER-positive MCF-7 cells expressing either WT ESR1 (as a control for elevated receptor levels), or the most frequent HBD mutants Y537N, Y537S, or D538G. Endogenous ER α (66 kDa), and an m-cherry-tagged exogenous receptor (~95 kDa) were visualized using immunoblot analysis (Fig. 1A), and growth responses were determined using soft agar growth assays. The most noticeable difference was the change in shape and larger size of the ESR1 mutant colonies under control conditions (-).

Quantification of colonies greater than 200 μ m showed that the control (-estrogen) growth of all mutant-expressing colonies was significantly enhanced (Fig. 1B). MCF-7 WT-expressing cells responded to Tam with a 38% and 53% reduction in soft agar growth with 0.1 or 1 μ M of 4-hydroxy-tamoxifen (designated Tam hereafter) treatment, respectively (Fig. 1C). In contrast, all HBD-ESR1 mutant-expressing cells exhibited reduced anti-proliferative responses to Tam, but with different absolute responses. Tam treatment of mutant cells showed less reduction in colony numbers (ranging from 42 to 13%) compared to WT-expressing cells even at the highest concentration used (Fig. 1C). MCF-7 Y537S ER-expressing cells consistently exhibited the least Tam-responsive phenotype. Similar data was obtained using another MCF-7 subline (designated BK) maintained separately in the laboratory (Figs. S1A and B). These data also suggest that there may be functional biologic differences between the three ESR1 mutations in their anti-proliferative responses to Tam.

We generated ESR1 mutant-expressing pools using two other ER-positive breast cancer cell lines, ZR-75B and T47D (Figs. S1C–F). Both of these models express high mutant levels. ZR-75B and T47D mutant cells responded to Tam treatment equivalent to WT-expressing cells, and colony size was not affected (data not shown). These results indicate that individual anti-proliferative responses to Tam may depend on cellular background, and that other tumor cell-intrinsic factors may be associated with response in cells expressing ESR1 mutants.

Activation of IGF-1 signaling in HBD-ESR1 mutants is associated with Tam response

Since response to Tam requires many cell-specific effectors, we next employed expression microarray profiling, 3-way ANOVA, and canonical pathway analysis using Ingenuity to identify pathways significantly associated with ESR1 mutant expression in MCF-7 compared to ZR-75B cells (Fig. 2A); darker orange bars are pathways most activated in MCF-7 mutant cells, and blue bars are deactivated pathways. The most significantly activated pathway was insulin growth factor-1 (IGF-1) signaling. A known mechanism of hormone resistance is enhanced cross-talk between ER and growth factor receptors, which can feedback and impact ER transcriptional activity. Based on this pathway analysis, we next investigated whether expression of the HBD-ESR1 mutations altered growth factor signaling, but no differences in Tam responses were detected with either epidermal growth

factor (EGF) or heregulin co-treatment (data not shown). However higher levels of phosphorylated IGF1R β , and phosphorylated insulin receptor substrate-1 (pIRS-1) were seen after IGF-1 treatment of MCF-7 mutant cells (Fig. 2B). Estrogen activation of IGF signaling served as a positive control in this experiment¹⁸. In contrast, we did not detect enhanced IGF1R β or IRS-1 phosphorylation in ZR-75B Y537S cells (Fig. 2C). Thus, activated IGF-1 signaling was specifically associated with mutant expression in MCF-7 cells exhibiting reduced anti-proliferative effects with Tam, implying that cross-talk with this signaling pathway may be involved. Activated IGF-1 signaling is a known mechanism affecting Tam response, and could be employed by ESR1 mutations as a proliferative escape mechanism in some cellular backgrounds.

We next used an IGF1R β inhibitor (GSK1838705A) that also inhibits signaling from the insulin receptor. Mutant ER transcriptional activity was efficiently blocked by Tam treatment, underscoring the limitation of only using transcriptional assays to monitor mutant function (Fig. 3A). The combination of Tam with GSK further enhanced the ability of Tam to block transcriptional activity, demonstrating functional ER cross-talk with IGF1R. We found that GSK treatment enhanced growth sensitivity to Tam in all cells (Fig. 3B). Of note, the growth of Y537S-ESR1 cells was significantly stimulated by Tam treatment in this experiment, and the IGF1R β inhibitor was able to block this agonist growth.

We performed knockdown of IGF1R β levels using two siRNAs to confirm these results, and knockdown enhanced Tam's anti-proliferative effects (Fig. 3C and D). These results suggest that specific blockade of IGF1R itself or other downstream signaling molecules may be required, along with hormonal therapy, in some mutant-expressing tumors.

Enhanced interactions between IGF1R and the Y537S ESR1 mutant

The MCF-7 Y537S model demonstrated the smallest anti-proliferative effects with Tam treatment, therefore this model was examined for protein interactions between IGF1R β and ER using immunoprecipitation coupled with immunoblot analysis. Input extracts were examined and confirmed higher levels of IGF1R β protein in MCF-7 mutant, but not in ZR-75B mutant cells; all cells were able to induce pIRS-1 with IGF-1 treatment (Fig. 4A). Higher constitutive binding was observed between the Y537S mutant and IGF1R β in MCF-7 (Fig. 4B), but not ZR-75B cells (Fig. 4C). This suggests that although all cells can express pIRS-1, there were diminished anti-proliferative effects in MCF-7 cells with elevated constitutive pIGF1R β levels. To further examine for interactions between IGF1R and ER, we also performed proximity ligation assays in MCF-7 and ZR-75B cells (Figs. 4D). Prominent interactions (red speckled staining, arrows) were seen in mutant-expressing cells compared to WT, but only in the MCF-7 cells. We conclude that the role of ESR1 mutations in Tam response may be dependent on a cellular background with elevated pIGF1R.

Everolimus and fulvestrant are effective at reducing growth of stable HBD-ESR1 mutant-expressing cells

Although a number of inhibitors targeting the IGF1R growth pathway have entered clinical trials, they have proven disappointing in randomized clinical trials, possibly because of the

from 12% for Y537N, 5% for Y537S, and 2% for D538G (Table 1). However allele frequencies were low in primary tumors, and are shown as the percent of mutant compared to WT ESR1 (Fig 6A), suggesting that these three mutations indeed represent a minor subpopulation within the primary tumor. We assessed the clinical impact of ESR1 mutations on outcomes in this cohort, and for patients with any (Y537N/S or D538G) mutation, patients with an ESR1 mutant exhibited slightly improved recurrence-free survival ([RFS], Fig 6B, $p=0.0118$), but no difference was seen in overall survival (OS, Fig. 6C). Effects of individual mutations on RFS or OS are shown in Supplementary Fig. S4. Thus the HBD-ESR1 mutations were not significantly associated with resistance to Tam as predicted by many preclinical studies. These results suggest that measurement of ESR1 mutation status as a single biomarker may not provide significant information in primary breast cancers treated with Tam.

Knockdown of PI3K regulatory units PIK3R1 and PIK3R3 enhances anti-proliferative effects of Tam

Undoubtedly, primary breast cancers are heterogeneous, and a combination of molecular alterations will define the functional consequences determining therapeutic sensitivity to hormone therapy. To explore other cell-intrinsic effectors of Tam response in mutant cells, we further examined the biology of two of the differentially-expressed IGF-1 pathway genes identified using comparative microarray analysis of WT and the Y537S mutant in MCF-7 vs. ZR-75B cells (Fig. 6D). Up-regulated IGF-1 pathway members the PI3K regulatory subunits 1 and 3 (PIK3R1 and PIK3R3). In addition to their roles in PI3K signaling, PIK3R1 and 3 can serve as adaptors for cellular signaling. PIK3R3 can physically interact with IGF1R and impact signaling²⁶, and naturally-occurring mutations in PIK3R1 activate PI3K/MAPK signaling and dictate sensitivity to MAPK pathway inhibitors²⁷. Since RNA levels of both PIK3R1 and 3 were elevated in MCF-7 but not ZR-75B Y537S mutant-expressing cells (Fig. 6D), we used siRNAs for specific knockdown of the 3R1 (p85) and 3R3 (p55) subunits, and examined effects on signaling and Tam response. Knockdown of PIK3R3 exhibited the greatest effect on reducing pIGF1R β levels in mutant cells, and affected proliferation of both WT and mutant cells (Fig. 6E and F). Knockdown of both regulatory subunits potentiated the anti-proliferative effects of Tam, especially in mutant-expressing cells. Thus, altered PI3K regulatory subunit expression may be cell-intrinsic factors associated with Tam response in breast cancer, especially in those patients expressing ESR1 mutants. These observations will require validation in clinical material from ESR1 mutant-positive patients.

DISCUSSION

The “rediscovery” of ESR1 mutations in metastatic breast cancer has reopened a number of important clinical questions about their role in acquired resistance to hormonal agents, and the metastatic spread of breast cancer²⁸. The most important clinical question of course is whether the mutations are targetable? To date, it is well-demonstrated that ER transcriptional activity associated with mutations in the HBD is hormone-independent, and that the mutations display different transcriptional responses to Tam and fulvestrant^{4,5,6,7}. Molecular modeling of the Y537S and D538G mutations show that they shift the receptor into an

agonist conformation, which may account for their elevated basal transcriptional activity⁷, and recently it has been shown these two mutations stabilize binding to co-activators and reduce binding affinity for Tam¹³. Using stable expression of three hot spot ESR1 mutations, we demonstrate that although the mutations reduce the absolute anti-proliferative effects of Tam depending on the cellular background, Tam is an effective agent to reduce anchorage-independent growth of cells expressing ESR1 mutations.

We employed microarray analysis to identify cell-intrinsic mediators associated with reduced Tam effects in MCF-7 compared to ZR-75B cells, and activation of the IGF-1 pathway was the most significant differentially-expressed pathway in Ingenuity analyses. Our data suggests that activation of IGF1R β was a key determinant of Tam response and was driven by cross-talk with the HBD-ESR1 mutations. Enhanced binding between the Y537S ESR1 mutant and IGF1R β was detected. Similarly, increased binding and cross-talk between IGF1R β /HER2 and the K303R ESR1 mutation has previously been reported^{29,30}. Enhanced cross-talk between WT ER and these growth factor receptors is a well-studied mechanism of hormone resistance in breast cancer^{31,32}. In K303R ESR1 mutant-expressing cells, components both upstream and downstream of the IGF1R pathway were altered with mutant expression²⁹. In ZR-75B cells expressing the HBD-ESR1 mutations Y537N, Y537S and D538G where differences in anti-proliferative effects to Tam were not observed, IGF-1 signaling was similarly not engaged. Thus, the ESR1 mutations may employ a common mechanism via cross-talk with the IGF1R pathway to escape hormone therapy in some cellular backgrounds.

Although previous preclinical studies have suggested that HBD-ESR1 mutants may be relatively resistant to the SERD fulvestrant^{4,5,33}, in this study, fulvestrant was very effective alone, or in combination with either an IGF1R or mTOR-targeting agent. It has been shown that the oral SERD AZD9496 inhibited the growth of a patient-derived xenograft (PDX) harboring the D538G mutation³⁴, and the SERM/SERD hybrid piperidoxifen inhibited the growth of a PDX with the Y537S mutation³⁵. An early report from the FERGI trial suggests that ESR1 mutations are not associated with differential PFS in the fulvestrant control arm, thus the mutations were not associated with resistance to fulvestrant³⁴. Thus this collective data strongly support the use of fulvestrant in metastatic patients harboring ESR1 mutations.

Another important clinical question is which component of the activated signaling cascade observed in ESR1 mutant tumors might be the most optimal therapeutic target for combination therapy with fulvestrant? Fulvestrant or exemestane combined with IGF1R inhibition was unsuccessful in a phase II trial of advanced breast cancer, which effectively halted the use of hormonal therapies combined with an IGF1R inhibitor in breast cancer³⁶. Our results showed that the combination of fulvestrant with the GSK inhibitor was very effective in ESR1 mutant cells with activated IGF-1 signaling. The assessment of the IGF-1 molecular signature and alternate IGF1R modulators, such as PIK3R3 also mediating IGF1R escape, within the context of a clinical trial, might improve patient selection for combined hormonal/IGF1R strategies in ESR1 mutant metastatic patients.

The PI3K/AKT/mTOR pathway is commonly altered in luminal tumors, and preclinical studies have shown that inhibition of these pathways can restore hormone sensitivity^{37,38,39}.

We show that this pathway is highly activated in ESR1 mutant-expressing cells, and everolimus in combination with either fulvestrant or Tam exhibited significant anti-proliferative effects in cells expressing the Y537N/S and D538G ESR1 mutations. Preliminary results from the BOLERO-2 trial suggest that metastatic patients harboring the D538G, but not the Y537S mutation respond to exemestane plus everolimus¹⁷. This is the first report in patients that selective ESR1 mutations might respond differentially to targeted therapies, and this significant finding awaits confirmation from other patient cohorts. A small phase II study of fulvestrant and everolimus in metastatic patients after AI treatment failure showed some efficacy in delaying fulvestrant resistance, and another phase II study of everolimus in combination with Tam also showed clinical benefit in AI-resistant metastatic patients⁴⁰, warranting further study of these combination in tumors with ESR1 mutations. It is interesting to note that elevated expression of PIK3R3, as was seen in MCF-7 YS cells, was reported to be a biomarker predicting for response to everolimus⁴¹. An important challenge of mTOR inhibition however is signaling feedback via elevated expression and activity of growth factor receptors, providing alternative survival pathways to evade therapy⁴². One could speculate that this could become particularly problematic in the treatment of ESR1 mutant tumors with constitutively activated pIGF1R.

The TCGA breast cancer next generation sequencing study did not detect ESR1 mutations in primary ER-positive tumors¹⁸; however, a recent report from Takeshita et al. using ddPCR reported a frequency of 2.5% in primary breast cancer⁴³. Emerging data using cell free circulating tumor DNA have reported ESR1 mutant frequencies at almost 40% in selected metastatic patients¹⁷. We also used a sensitive ddPCR technique to detect the three HBD-ESR1 mutations, and report a high frequency of these mutations in primary tumors. The low allele frequency of the ESR1 mutations in our cohort suggests that the mutations exist in only a minor subpopulation of the primary tumor. Using single cell sequencing it has been demonstrated that mutations in breast cancer occur at low frequencies and evolve gradually over time⁴⁴. In a small series of patients monitored for circulating tumor DNA, it has been shown that ESR1 mutations are rarely selected during adjuvant AI therapy, but are commonly selected for during AI therapy for metastatic disease. Since AI therapy might provide a selective advantage to the HBD-ESR1 mutations, our data suggest that the use of targeted ESR1 mutation sequencing might be warranted earlier in patients with advanced breast cancer. Since the ESR1 mutations might also confer enhanced metastatic potential and/or aggressive biological attributes, testing of this possibility in archived material from adjuvant studies is warranted.

MATERIALS AND METHODS

Reagents, Hormones, and Antibodies

4-OH-tamoxifen (Tam), 17 β -estradiol, insulin growth factor-1 (IGF-1) and puromycin were purchased from Sigma (St. Louis, MO, USA). GSK1838705A (GSK) and everolimus were obtained from Selleck Chemicals (Houston, TX, USA). MEM, RPMI 1640, DMEM, L-glutamine, penicillin/streptomycin, MEM non-essential amino acids and SeaPlaque™ Agarose were from Lonza (Walkersville, MD, USA). Fetal bovine serum was obtained from Gemini Bio Products (West Sacramento, CA, USA). SuperScript III reverse Transcriptase,

qPCR probes (IGF1R β and GAPDH), and lipofectamine LTX was provided by Life Technologies (Grand Island, NY, USA). Antibodies were: ER α (Vector Laboratories, Burlingame, CA, USA); total IGF1R, IRS-1, mTOR, phosphorylated IGF1R^(Tyr1131), mTOR^(Ser2448), pS6^(Ser240, 244), p85 and p55 (Cell signaling Technology, Beverly, MA, USA); IRS-1^(Tyr612) (Invitrogen, Carlsbad, CA, USA); and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Goat anti-mouse and anti-rabbit secondary antibodies were from Amersham Biosciences (Piscataway, NJ, USA). The Renilla Luciferase assay kit was from Promega (Madison, WI, USA). GFP-nAB beads were from Allele Biotechnology (San Diego, CA, USA).

Plasmids

Y537N, Y537S, and D538G-ER α constructs were generated using QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA, USA) to make the indicated point mutations in LV111-mCherry-ER α (purchased from Genecopoeia, Rockville, MD, USA). The primer sequences for construction of the point mutations were as follows: Y537N, 5'-cgtggtgccccctcatgacctgctgctggag-3'; Y537S, 5'-cgtggtgccccctctctgacctgctgctggag-3'; D538G, 5'-cgtggtgccccctcatgccctgctgctggag-3' with the mutated nucleotides italicized and underlined. The entire nucleotide sequence of the construct then was verified using Sanger Sequencing.

Empty vector (EV), IGF1R siRNAs, PIK3R1 and PIK3R3 siRNAs, were provided by Addgene (Cambridge, MA, USA).

Cell Culture

MCF-7 and MCF-7 BK cells were grown in MEM; T47D and ZR-75B cells (generously obtained from Dr. Marc Lippman) in RPMI-1640, HEK293 in DMEM without sodium pyruvate were grown supplemented with 10% FBS, 100 I.U./ml Penicillin, 100 μ g Streptomycin, and 0.1mM non-essential amino acids. All stable clones were maintained in puromycin (1 μ g/ μ l).

Stable Clones

Transduced cells were generated as previously described⁴⁵. A growth disadvantage was observed during selection, especially with the Y537N and Y537S clones,.

Transfection Assays

Transfection for ERE-luciferase assays, siRNA, immunoblot, coimmunoprecipitation and PLA assays was performed using the lipofectamine reagent as recommended by the manufacturer.

Proximity Ligation Assays

Proximity ligation assays was performed using Duolink detection reagent kits (Sigma-Aldrich, St. Louis, MO, USA). Fluorescence was detected using a Leica confocal microscope TCS SP5 (Leica Microsystems, Buffalo Grove, IL, USA) and Leica Application Suite Software (LAS, Wetzlar, Germany, EU).

Cell Extraction, Immunoblot and Coimmunoprecipitation Analysis

Cells were cultured in regular media or were starved in 5% charcoal stripped-serum media for 24h, and were treated with IGF-1 50ng/ml for 15' and E2 100nM for 2h, GSK 1 μ M, everolimus 100nM and Tam 100nM for 5 days (immunoblot analysis), with IGF-1 50ng/ml for 2h (coimmunoprecipitation analysis) before lysis. Immunoblot analyses were performed as previously described⁴⁵. Coimmunoprecipitation analyses were performed using the GFP-nAb beads.

Cell Proliferation Assays

Cell proliferation was measured using a soft agar anchorage-independent assay as previously described⁴⁵.

Expression Microarray Analysis

Cells were plated in regular media for 24 hours and then treated for another 24 hours with Tam 100nM. RNA was extracted using the RNeasy micro kit (Qiagen, Valencia, CA, USA). Labeled cRNA was hybridized onto Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix Inc. Santa Clara, CA, USA) with three chips each. Expression values were estimated using RMA method with Partek software (<http://partek.com/>). Three-way ANOVA with contrasts were run using Partek to find differentially-expressed genes. Differentially-expressed genes with FDR (Fold Discovery Rate) = 0.01 and Fold=3 was then used for Ingenuity Pathway Analysis (<http://www.ingenuity.com/>).

Wound-Healing Scratch Assays

Cell monolayers were scraped and wound closure monitored over 12–24h, and cells fixed and stained with Coomassie brilliant blue. Pictures were taken at 10X magnification using phase-contrast microscopy. The rate of wound healing was quantified from the images using Scion Image Program.

Mutation Detection using ddPCR

DNA was isolated from archived, formalin fixed paraffin-embedded patient samples and amplified using a QX100 ddPCR system. In this study we used invasive breast cancer obtained from women in the United States, and maintained in an archived tumor bank at Baylor College of Medicine. Patients were diagnosed between 1973 and 1993 and treated with mastectomy or lumpectomy plus axillary dissection, with or without postoperative radiation therapy. All patients had been treated with adjuvant Tam monotherapy¹¹. The fraction of positive droplets determined the concentration of the target molecules in the sample; any drop was considered positive. Samples were analyzed by fractional abundance (molecules of mutation/molecules of WT). Specific assays for ESR1 Y537N, Y537S or D538G were designed and optimized using MCF-7 stable expression cell lines. FAM was used for Y537S, Y537N and D538G mutations, and HEX for wild type.

Sequences for amplicons generated: Y537N WT:

TACAGCATGAAGTGCAAGAACGTGGTGGCCCTCTATGACCTGCTGCTGGAGATGC
TGGACG; Y537N

Mutant:GTACAGCATGAAGTGCAAGAACGTGGTGCCCTCAATGACCTGCTGCTGG
 AGATGCTGGACG; Y537S WT:
 GTACAGCATGAAGTGCAAGAACGTGGTGCCCTCTATGACCTGCTGCTGGAGATG
 CTGGACGCCC; Y537S Mutant:
 TACAGCATGAAGTGCAAGAACGTGGTGCCCTCTCTGACCTGCTGCTGGAGATGC
 TGGACGCCC; D538G WT:
 GTACAGCATGAAGTGCAAGAACGTGGTGCCCTCTATGACCTGCTGCTGGAGATG
 CTGGACGCCC; D538G Mutant:
 GTACAGCATGAAGTGCAAGAACGTGGTGCCCTCTATGGCCTGCTGCTGGAGATG
 CTGGACGCCC.

Statistical Analyses

Data were analyzed by student's *t*-test using GraphPAD Prism5 software (GraphPad Software, Inc. San Diego, CA, USA). Standard deviations (SD) are shown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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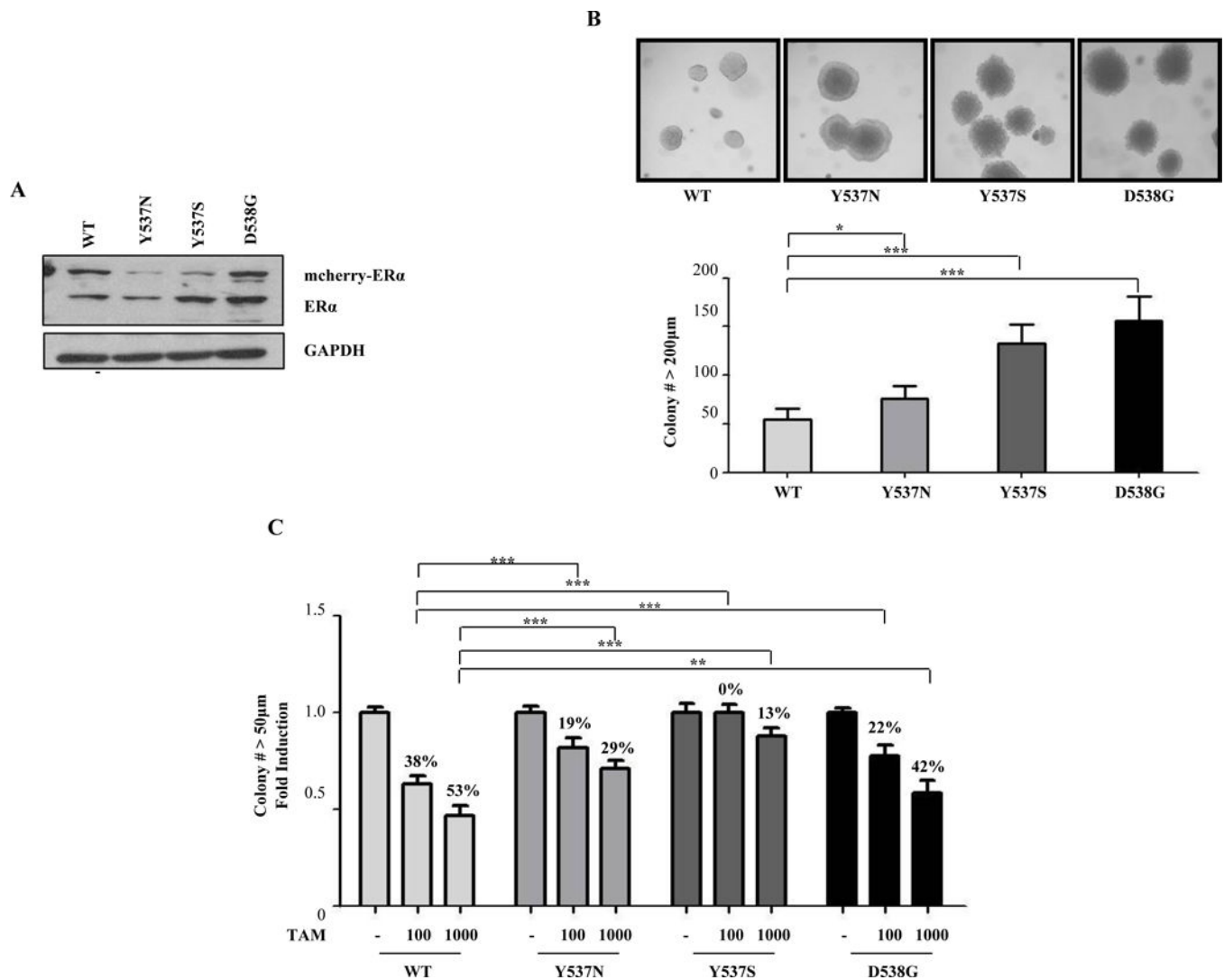


Figure 1. HBD-ESR1 mutations influence Tam response

a, immunoblot analysis of detect ER expression in MCF-7 stable clones. GAPDH was used as loading control. **b**, Representative photographs of three different well plates captured from soft agar assays showing higher number of large colonies (200 μ m) in mutant-expressing cells. Experiments were performed in triplicate and error bars indicate SD.

* $P < 0.05$; *** $P < 0.001$. **d**, Cells were plated for soft agar assays, and then treated with vehicle or two doses of Tam (100nM or 1000nM). Percentage of growth reduction with Tam treatment is shown. Experiments were performed in triplicate and error bars indicate SD. ** $P < 0.01$; *** $P < 0.001$.

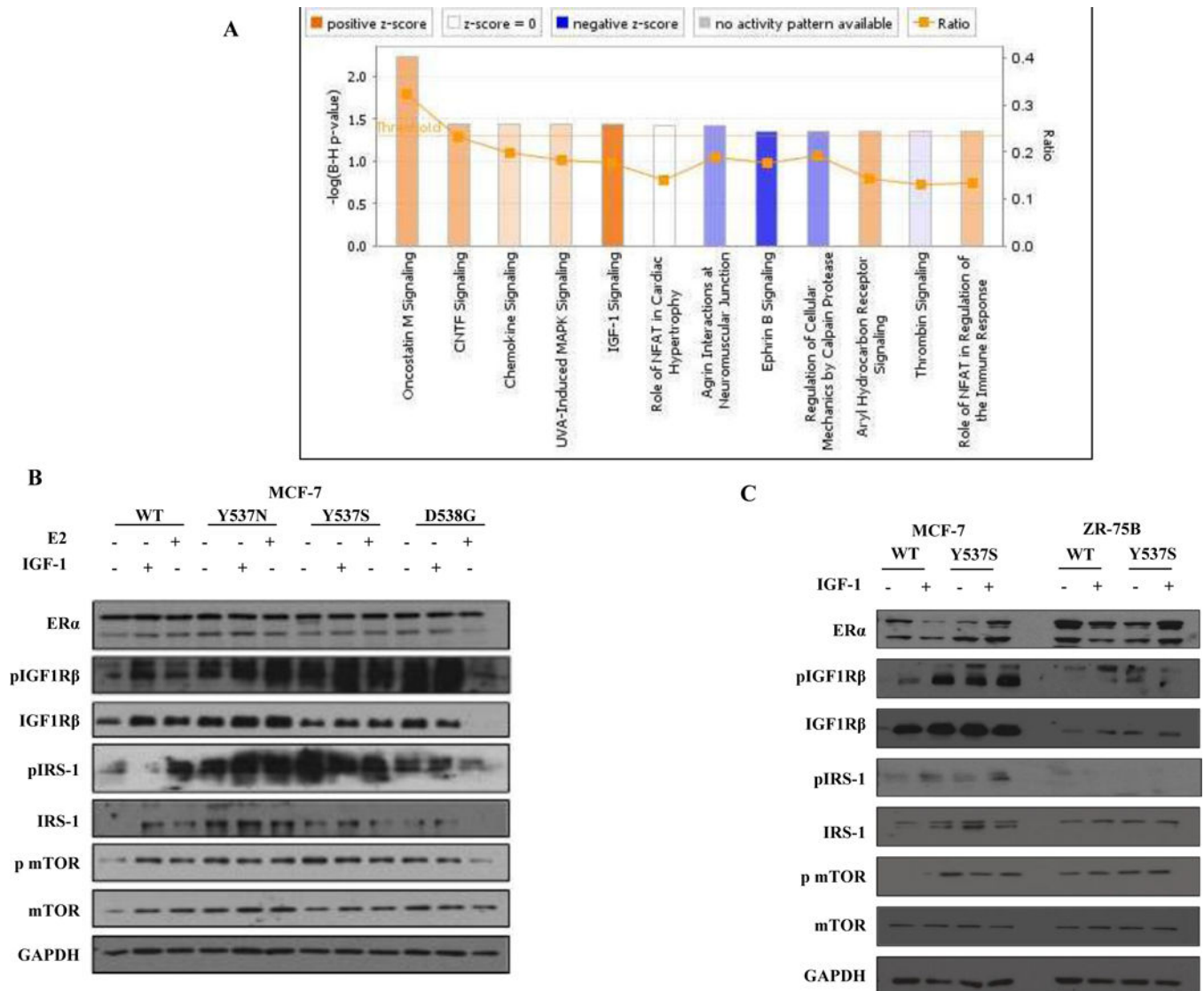


Figure 2. IGF-1 signaling pathway activation in HBD-ESR1 mutants

a, Ingenuity Pathway Analysis (IPA) to identify activation of signaling pathways in mutant MCF-7 vs. ZR-75B. **b**, Total cellular extracts were analyzed for phosphorylation and expression of ER, IGF1R β , IRS-1, and mTOR; GAPDH was used as a loading control. Immunoblots show a representative example of three experiments. **c**, Total cellular extracts were analyzed for phosphorylation and expression of ER α , IGF1R β , IRS-1, and mTOR; GAPDH was used as a loading control. Immunoblots show a representative example of three experiments.

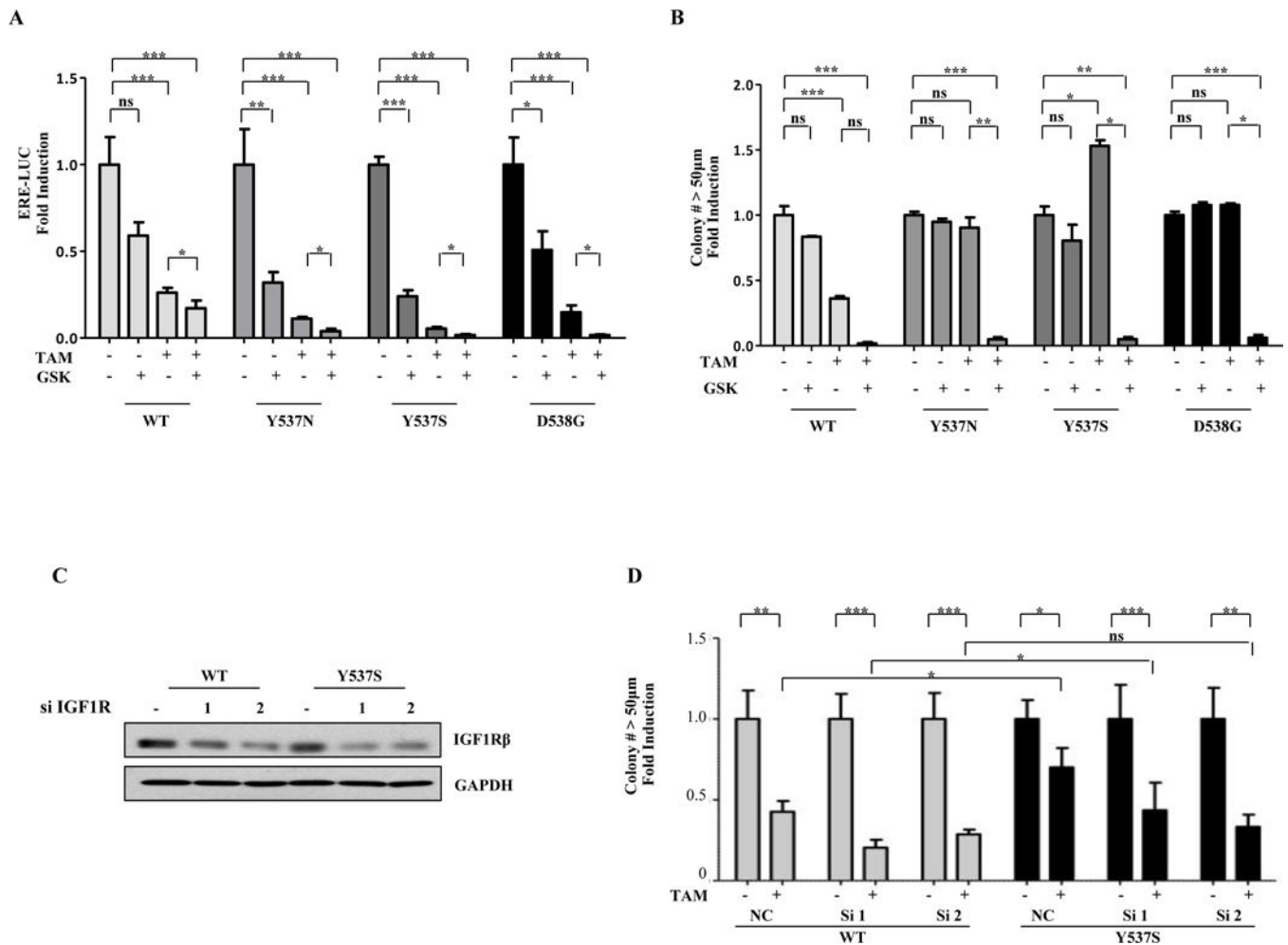


Figure 3. Specific blockade of the IGF1R pathway restores anti-proliferative effects of Tam in mutant-expressing cells

a, ERE-luc assays in MCF-7 cells transiently transfected with WT-, Y537N-, Y537S-, or D538G-ER plasmids. Experiments were performed in triplicate and error bars indicate SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **b**, Experiments were performed in triplicate and error bars indicate SD. ns, not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **c and d**, Cells were transfected for 24 hours with two different IGF1R β siRNAs (100nM) and were maintained for 14 days in culture. 10 μ g of protein were loaded per well and the blot was probed with antibodies detecting IGF1R β ; GAPDH was used as a loading control (**c**), Same cells were plated for soft agar assay and then treated with vehicle (-) or with Tam (100nM). Experiments were performed in triplicate and error bars indicate SD. ns, not significant, * $P < 0.5$.

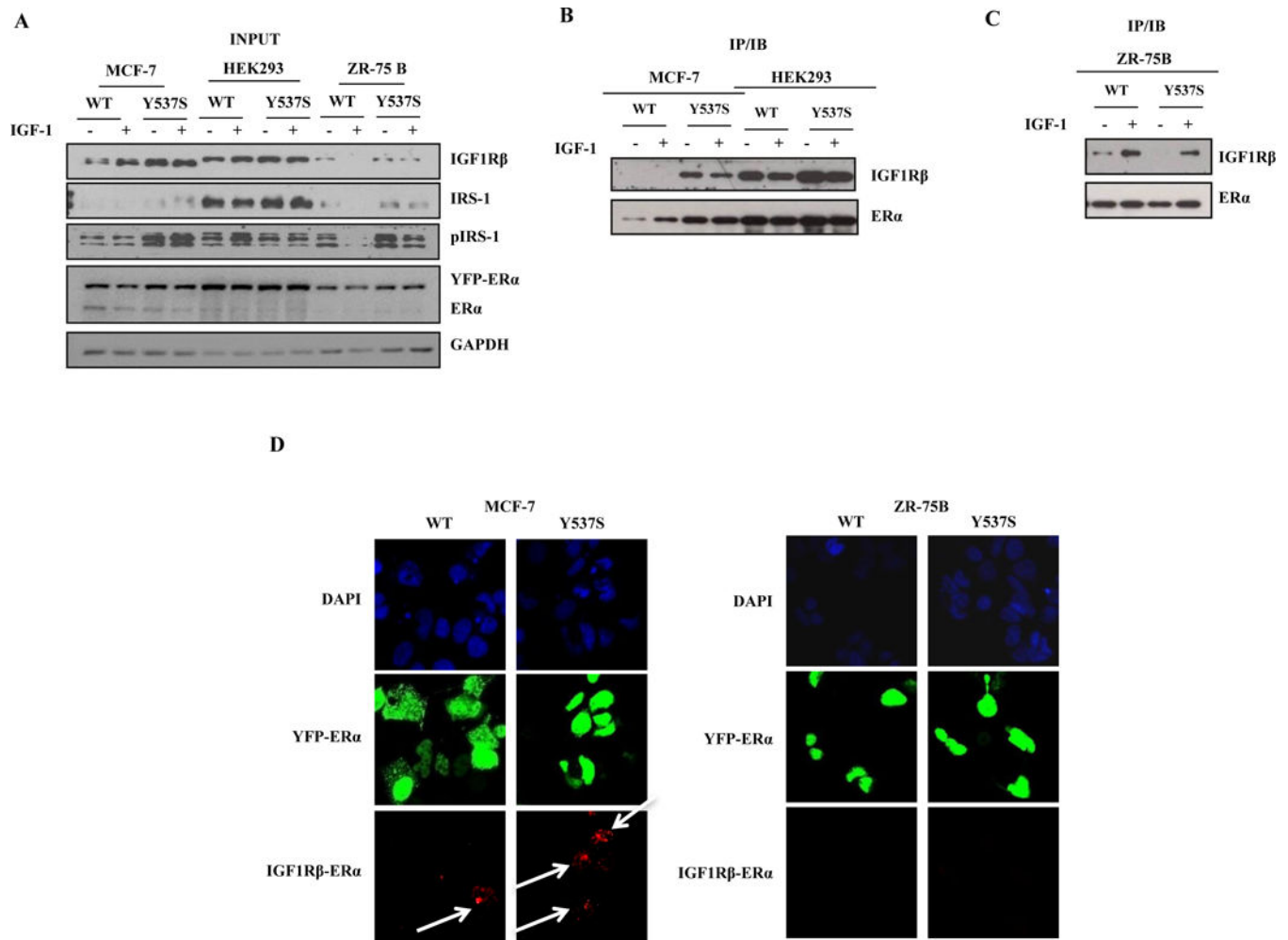


Figure 4. MCF-7 Y537S cells show the lowest anti-proliferative response to Tam through increased interaction between IGF1R/ER

a, b, and c, Immunoblot analysis to detect expression of IGF1R β , pIRS-1, IRS-1 and ER in input extracts; GAPDH was used as a loading control (**a**). Coimmunoprecipitation assays of IGF1R/ER interactions (**b and c**). Duolink staining (red) demonstrated binding between IGF1R and ER in MCF-7(**d**) and ZR-75B cells (**e**). Images are representative of three different experiments.

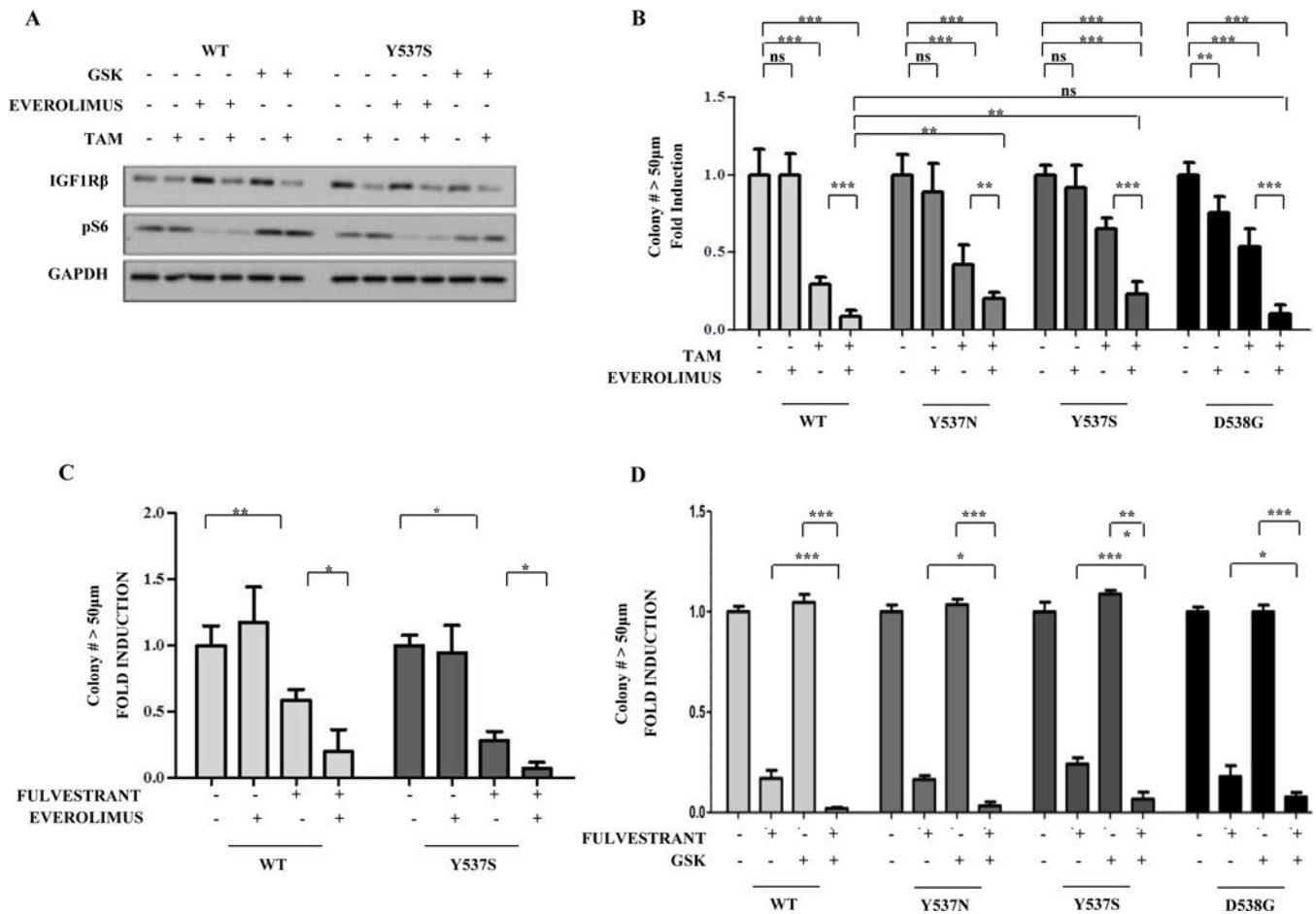


Figure 5. Fulvestrant in combination with everolimus or GSK enhances anti-proliferative effects in mutant-expressing cells

a. Immunoblot analysis to detect IGF1R β and pS6 levels; GAPDH was used a loading control. Immunoblots show a representative example of three different experiments. **b.** Cells were plated in soft agar assay and then treated with vehicle (-), Tam (100nM), or everolimus (1nM). Experiments were performed in triplicate and error bars indicate SD. ns, not significant. *P<0.05; **P<0.01; ***P<0.001. **c.** Cells were plated in soft agar assay and then treated with vehicle (-) or with fulvestrant (100nM) and everolimus (1nM). Experiments were performed in triplicate and error bars indicate SD. *P<0.05. **d.** Cells were plated in soft agar assay and treated with vehicle (-), fulvestrant (100nM) and/or GSK (1 μ M). Experiments were performed in triplicate and error bars indicate SD. *P<0.05, ***P<0.001.

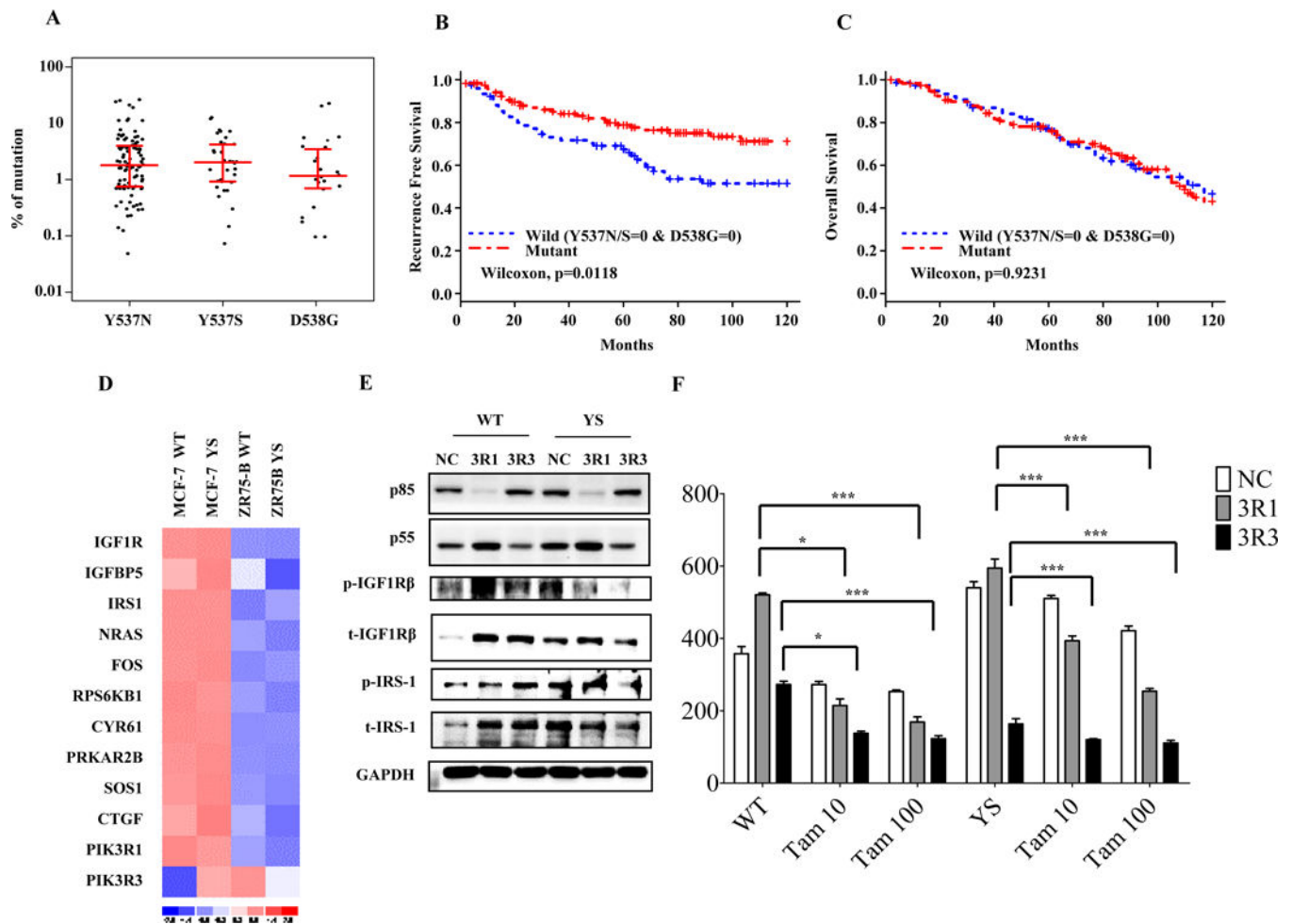


Figure 6. ESR1 mutation do not predict outcomes in patient treated with adjuvant Tam
a, ddPCR analysis showing allele frequencies of three HBD-ESR1 mutations in primary breast cancers. **b and c**, Kaplan–Meier analysis of RFS (**b**), and OS (**c**). **(d)** Heatmaps of mRNA levels of genes involved in IGF-1 signaling pathway comparing MCF-7 and ZR-75B stable. Immunoblot analysis to detect phosphorylation and total protein expression of IGF1R β , pIRS-1, p85 and p55; GAPDH was used as a loading control (**e**). Immunoblot of cells transduced with siRNAs for p55 and p85. **(f)**. Cells were also plated in soft agar assays and treated with vehicle (-) or with Tam (10 and 100nM). Experiments were performed in triplicate and error bars indicate SD. ns, not significant. * $P<0.05$; *** $P<0.001$.

Table 1

Mutation frequency in invasive breast tumors treated with Tam

Mutation	Frequency (%)	Number of tumors
Y537N	12	74/195
Y537S	5	29/199
D538G	2	4/195

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