Research article

XBP1 increases transactivation of somatic mutants of ESR1 and loss of XBP1 reverses endocrine resistance conferred by gain-of-function Y537S ESR1 mutation

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ABSTRACT

Somatic mutations of the estrogen receptor 1 gene (ESR1) is an emerging mechanism of acquired resistance to endocrine therapy in hormonal breast cancers. Hotspot point mutations in the ligand-binding domain of estrogen receptor α (ER) and genomic rearrangements producing ESR1 fusion genes are the two major types of mutations that are associated with endocrine resistance. The crosstalk between X-box binding protein 1 (XBP1), a key transcription factor of the unfolded protein response (UPR) and ER signalling creates a positive feedback loop that results in increased expression of XBP1 in ER-positive breast cancer. Here we report that XBP1 co-operated with point mutants (Y537S, D538G) and fusion mutants (ESR1-YAP1, ESR1-DAB2) of ESR1 to increase their transcriptional activity. XBP1 was required for optimal expression of estrogen-regulated genes, and up to 40% of XBP1-dependent genes were estrogen-responsive genes. Knockdown of XBP1 in genome-edited MCF7 cells expressing Y537S mutant reduced their growth, re-sensitized them to anti-estrogens and attenuated the constitutive and estrogen-stimulated expression of estrogen-regulated genes. Our study provides a rationale for overcoming endocrine resistance in breast cancers expressing ESR1 mutation by combining the XBP1 targeting agents with anti-estrogen agents.

1. Introduction

Around 75% of breast cancers are positive for estrogen receptor α (ER), and endocrine therapies remain the standard treatment options for their management. However, one-third of cancer patients treated with endocrine therapies acquire resistance leading to disease progression and death [1, 2] Dysregulated expression of ER and its downstream signalling pathways are important determinants of therapeutic response of ER-positive breast cancers to anti-estrogens. Reported mechanisms for the aberrant estrogen (E2) signalling includes somatic mutations of ER, hyperactivity of co-activator proteins, dysregulation of growth factor receptor signalling and the activation of unfolded protein response (UPR) [3, 4, 5]. Several studies have reported genomic alterations at the ESR1 locus, which is an emerging mechanism that drives endocrine resistance in ER-positive breast cancer [2, 6, 7]. Hotspot point mutations in the ligand-binding domain of ESR1 have been found in up to 40% of metastatic, endocrine-resistant

ER-positive breast cancer patients [6]. Two most prevalent ESR1 missense mutations (Y537S and D538G) show constitutive, estrogen-independent transcriptional activity, and partial-resistance to hormonal therapy [1, 8, 9]. Further, several other ESR1 fusion mutations have also been identified including ESR1-DAB2 [10] and ESR1-YAP1 [11], that are implicated in advanced, endocrine-resistant breast cancer [12]. Both ESR1-YAP1 and ESR1-DAB2 fusion transcripts demonstrate similar structural rearrangements that arise by the inter-chromosomal translocation of ESR1 gene with Yes Associated Protein 1 (YAP1), and disabled homolog 2 (DAB2), respectively [12]. Both fusion proteins exhibit ligand-independent transactivation function, and ESR1-YAP1 is associated with increased cell motility, and metastatic potential [10, 11]. Resistance conferred by both types of ESR1 mutations poses a significant challenge to treat metastatic, ER-positive breast cancer. There is an urgent need to identify new effective treatments, in order to best treat metastatic, ER-positive breast cancers harbouring ESR1 mutations.

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In response to conditions that increase protein synthesis and secretion by the cell, an intracellular signalling pathway, the unfolded protein response (UPR) is activated [13, 14]. IRE1 is an evolutionarily conserved sensor of UPR that has protein kinase and RNase activity [15]. Upon activation, IRE1 undergoes auto-phosphorylation, inducing a conformational change that triggers its RNase activity [15]. Activated IRE1 catalyses an unconventional cytoplasmic mRNA-splicing reaction, that introduces a change in the reading frame of the XBP1 mRNA [15]. Spliced XBP1 mRNA produces an active and stable transcription factor (XBP1s), whereas unspliced XBP1 mRNA generates a short-lived protein that lacks transactivation function [15, 16]. The increased expression of XBP1 is associated with poor prognosis and high grade tumours in ER-positive breast cancer [17]. Several groups, including ours, have previously shown a close interaction between the XBP1 and estrogen signalling [18, 19, 20]. Over expression of XBP1s in MCF7 breast cancer cells causes estrogen-independent growth and resistance to endocrine therapy [21]. The endocrine-resistant breast cancers show increased expression of XBP1s [22]. The obligate requirement of IRE1-dependent splicing to generate XBP1s has been exploited to generate chemical inhibitors to selectively block the production of XBP1s protein [22]. Several inhibitors have been reported that selectively inhibit the RNase activity of IRE1 and blocks the splicing of XBP1 mRNA [22]. Blocking XBP1s expression by STF-083010 has been shown to re-sensitize the tamoxifen-resistant MCF7 cells in a xenograft model [23]. However, the therapeutic potential of targeting XBP1 to overcome endocrine resistance conferred by gain-of-function mutants of ESR1 is not yet reported. Here we have focused on the most commonly occurring ER mutation (Y537S) and evaluated the efficacy of targeting the XBP1 signalling in combination with endocrine therapy for abrogating the endocrine resistance.

In this study, we report that XBP1 can co-operatively increase the transactivation function of ESR1 mutants in a ligand-independent manner. We show that XBP1 is required for the optimal induction of estrogen-responsive genes which are upregulated in Y537S and D538G cells. Furthermore, knockdown of XBP1 attenuated the proliferation of Y537S expressing MCF7 cells and sensitized them to tamoxifen. Our results suggest that targeting of XBP1 in combination with endocrine therapy may improve clinical outcomes in breast cancer patients harbouring ESR1 mutations.

2. Materials and methods

2.1. Cell culture and reagents-

HEK 293T cells were from Indiana University National Gene Vector Biorepository (Indianapolis, IN, USA). MCF7 cells was obtained from ECACC (Salisbury, UK). MCF7-Y537S and MCF7-D538G cells were a kind gift from Dr. Steffi Oesterreich (University of Pittsburgh, USA) [8]. Cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). MCF7-Y537S and MCF7-D538G mutant cells were cultured using DMEM and 5% FBS. Regular FBS and charcoal-stripped serum (CSS) were from Labtech international. All other reagents were from Sigma unless otherwise stated. For estradiol treatment, MCF7 Y537S and MCF7 D538G cells were cultured in phenol red free DMEM containing 5% CSS, and after synchronization for 3 days, cells were treated with estradiol (10 nM) for 24h.

2.2. Plasmid constructs

3X ERE-LUC (Cat# 11354), pcDNA-HA-ER WT (Cat# 49498), pcDNA-HA-ER Y537S (Cat# 49499), pcDNA-HA-ER D538G (Cat# 49500), β-galactosidase (Cat# 83943), packaging plasmids psPAX2 (Cat# 12260) and PMD2.G (Cat# 12259) were from addgene. ESR1-DAB2 plasmid was a kind gift from Dr. Adrian Lee (University of Pittsburgh, USA) and ESR1-YAP1 plasmid was a kind gift from Dr. Charles Foulds (Baylor College of Medicine, USA). Lentiviral PLKO control and XBP1-targeting shRNA plasmid (TRCN0000019805) were from Sigma (Wicklow, Ireland).

CRISPR guide RNA plasmids targeting XBP1 were purchased from Origene (Cat# KN201959).

2.3. Cell transfection and luciferase reporter assay

Luciferase reporter assays were performed as described previously [8]. MCF7 and 293T cells were routinely maintained in DMEM media containing 10% FBS in 37 °C humidified incubator and 5% CO_2. Before transfection, cells were plated in either a 6-well plate or 12-well plate using phenol red-free DMEM media containing 5% CSS. After 24h of plating, cells were transfected with either control or wild type ER plasmid (450 ng) or indicated mutant ER plasmid (450 ng) together with ERE luciferase reporter plasmid (450 ng). For XBP1s co-transfection, 150 ng of either wild type or mutant ER plasmid, 300 ng of ERE luciferase plasmid, 300 ng of XBP1s expression plasmid and empty vector was used to adjust the total DNA concentration. For each transfection, 100 ng of β-galactosidase plasmid was used to normalise the transfection efficiency. MCF7 cells were transfected using Turbofect (Thermofisher) and 293T cells were transfected using JetPEI (Polypus transfection, VWR) and at 24h of pot-transfection, cell lysates were analysed for reporter assay. Firefly activity was measured using a luciferase assay system (Promega). The β-galactosidase assay was performed using the same lysates to normalise the transfection efficiency.

2.4. Generation of XBP1 shRNA and knockout clones

The details of the lentivirus generation has been described previously [24]. MCF7-Y537S cells were transduced with control (PLKO) and XBP1 shRNA lentivirus and selected using puromycin (1 μg/ml). MCF7 XBP1 knockout cells were generated using CRISPR-CAS9 gene knockout strategy. MCF7 cells were transfected with two gRNA plasmids (5′-ACTTTAGGGGTCCCGTCGGC-3′ and 5′-CCCGTCGGCCGGGTTCGGCG-3′) targeting XBP1 using 4D nucleofector (Lonza) and Amaxa nucleofector solution (Lonza) according to the manufacturer's instructions. Pool cells were selected using puromycin (1 μg/ml), single cell clones were isolated and positive clones were confirmed using western blot and PCR.

2.5. RNA extraction, reverse transcription, and qRT-PCR

This has been described previously [18].

2.6. MTS cell proliferation assay

Cell proliferation using MTS assay has been described previously [24]. Briefly, cells (2,000/well) were plated in 96-well plate using complete DMEM media containing 5% FBS, and the following day, cells were either untreated or treated with the indicated compounds. Absorbance at 490 nm was measured at required time points.

2.7. Colony formation assay

Cells (500 cells/well) were plated in a 6-well plate using complete DMEM media containing 5% FBS. After two weeks, cells were washed with PBS and fixed with 10% formaldehyde for 5 min followed by staining with 0.05% crystal violet for 10 min. The number of colonies was determined using Image J software.

2.8. Western blotting

The western blotting has been described previously [25]. The nitrocellulose membrane was blocked with 5% non-fat dry milk in PBS/0.05% tween and incubated with primary antibodies, ER (N-terminus) at 1:2, 000 (Merck Millipore), XBP1s at 1:1,000 (Biolegend), and β-Actin at 1:5, 000 (Sigma). Following day, after washing, membranes were incubated

with secondary antibodies for 2h at room temperature, and finally, signals were detected using the chemiluminescent substrate (Perkin Elmer).

2.9. Statistical analysis

Data analysis was performed using Graph Pad Prism 5. Data presented as mean $\pm$ SD. Two-tailed unpaired t-test was used to determine the statistical significance between independent groups, and P < 0.05 was considered as statistically significant.

3. Results

3.1. Estrogen-independent transcriptional activity of ESR1 mutants

To confirm the ligand-independent activity of ESR1 mutants, the reporter assays were performed in steroid-free medium. We co-transfected (WT, Y537S, D538G, ESR1-DAB2, and ESR1-YAP1) ER constructs into HEK 293T cells with an estrogen-response element-luciferase (ERE-LUC) reporter plasmid. The wild-type (WT) ER showed minimal ERE-LUC reporter activity in steroid-free conditions but all the ER mutants showed significant, constitutive activation of the ERE-LUC reporter (Figure 1A). Next, we tested the ability of ER mutants to drive estrogen-independent transcription in the context of ER-positive breast cancer. We found that ERE transcriptional activity of all the mutant constructs was significantly higher than the wild-type ER in MCF7 cells (Figure 1B). Next, we determined the expression of all the ER proteins to assess whether the observed increase in transactivation function of mutant ER proteins is due to increased steady-state protein levels or activity. We observed that the expression of both the point-mutants was comparable to the wild-type ER protein, but the expression of both fusion-mutants was significantly higher (Lower panel, Figure 1A, B and SF1). Y537S among the point-mutants and ESR1-YAP1 among the fusion-mutants showed higher estrogen-independent activity in both cell lines.

3.2. XBP1s augments the transcriptional activity of gain-of function ESR1 mutants

XBP1s expression can confer estrogen-independence and resistance to endocrine therapy in ER-positive breast cancer [21, 26]. Next, we evaluated the effect of XBP1s co-expression on the activity of mutant ER

proteins. For this purpose, MCF7 cells were co-transfected with the ERE-LUC reporter and (WT, Y537S, D538G, ESR1-DAB2, and ESR1-YAP1) ER constructs, in the absence and presence of XBP1s. As previously reported, the co-expression of XBP1s significantly increased the transcriptional activity of wild-type ER (Figure 2A). We observed that XBP1s increased the transactivation function of both the point and fusion mutants of ESR1. Surprisingly, XBP1s expression alone also showed an increase in ERE-LUC activity, likely due to the augmentation of activity of the endogenous ER in MCF7 cells (Figure 2A). Furthermore, we analysed the expression of all the ESR1 mutants, alone or in the presence of XBP1s. We found that XBP1s co-expression had no significant effect on the expression of wild-type and (Y537S and D538G) point mutants of ER (Figure 2B and SF2). In contrast, we observed an increase in the expression of (ESR1-DAB2 and ESR1-YAP1) fusion mutants in the presence of XBP1s (Figure 2B and SF2). Our results show that increased luciferase activity upon co-expression with ESR1 mutants was accompanied by the increased expression of fusion mutants as compared to wild type ER, but the expression of ESR1 point mutants was comparable with wild type ER.

3.3. XBP1 is required for induction of estrogen-responsive genes constitutively upregulated in cells expressing Y537S and D538G mutants

Estrogen stimulation induces the expression of the XBP1s in ER-positive breast cancer cells and potentiates the ligand-independent transactivation function of ER [26, 27]. We identified estrogen-responsive genes that are regulated by XBP1 from the overlap of genes downregulated in T47D-XBP1 shRNA cells (GSE49953) and estrogen-regulated genes [28]. We found that among the 442 XBP1-dependent genes up to 172 (40%) of them were estrogen-responsive (Figure 3A). Next, we compared the estrogen-mediated upregulation of identified genes such as GREB1, PGR, and H19 in control and XBP1 knockout MCF7 cells. XBP1 knockout sub-clones of MCF7 cells were generated using the CRISPR-CAS9 system. XBP1 was required for basal expression as well as optimal estrogen-mediated induction of GREB1, PGR, and H19 (Figure 3B). In agreement with the ERE-LUC assays, genome-edited MCF7 cells expressing Y537S or D538G mutants showed a significant increase in the basal expression of ER-responsive genes such as GREB1, H19, and XBP1 in steroid free conditions as compared to control cells expressing
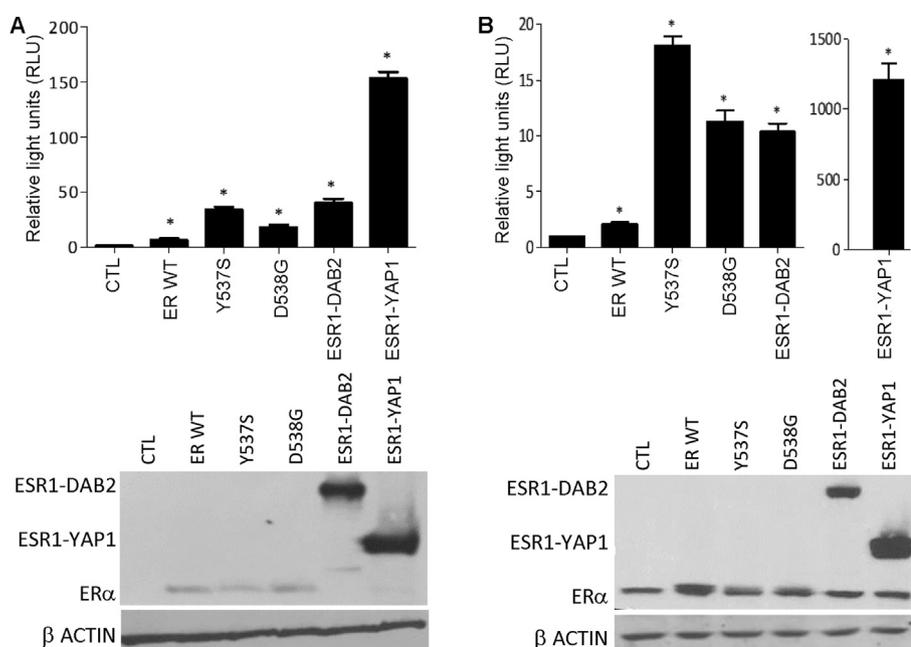
Figure 1. Ligand-independent transcriptional activity of ESR1 mutants. (A) Upper panel, HEK293T cells were transfected with ERE-LUC and indicated plasmids, 24h post-transfection, reporter assays were performed. Normalised LUC activity is presented as mean $\pm$ SD (n = 3). Lower panel, 24h post-transfection cell lysates were analysed by western blotting using antibodies against ER and β-actin. B) Upper panel, MCF7 cells were transfected as in A, 24h post-transfection reporter assay was performed. Normalised LUC activity is shown as mean $\pm$ SD (n = 3). Lower panel, 24h of post-transfection with indicated plasmids, cell lysates were analysed by western blotting using antibodies against ER and β-actin. *p < 0.05, two-tailed unpaired t-test. ERE, estrogen response element; LUC, luciferase; ER, estrogen receptor alpha. Uncropped full-length pictures of Western blotting membranes presented in the Supplementary Figure 1.
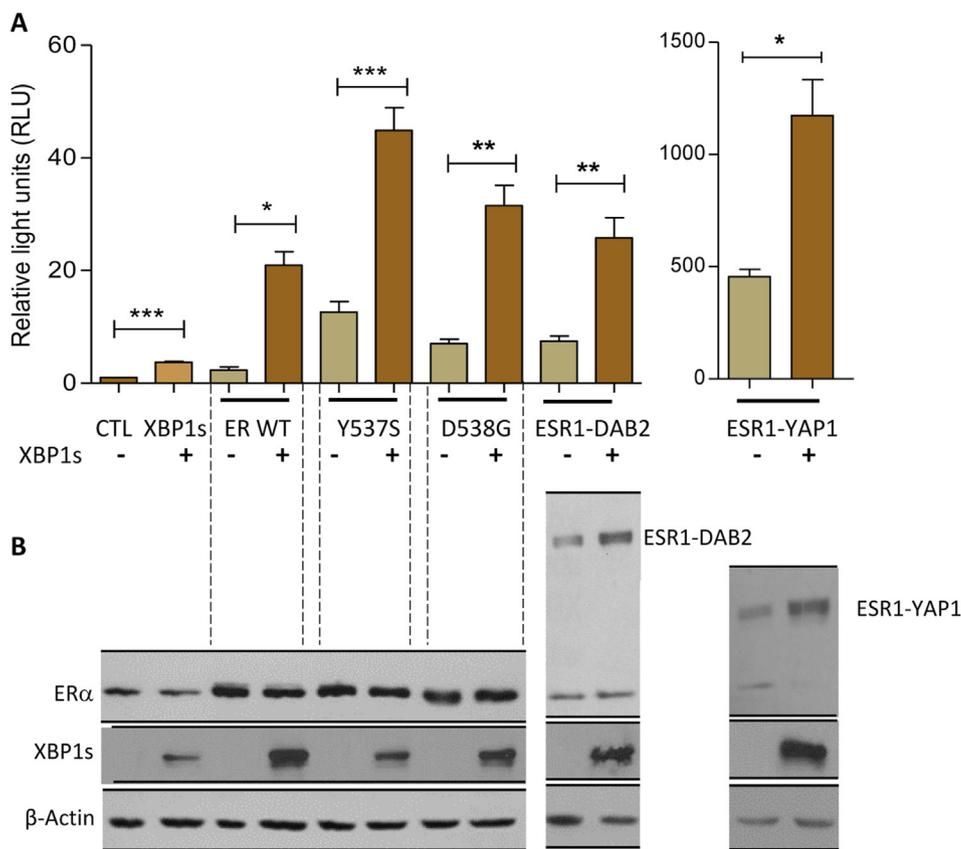
Figure 2. XBP1s enhances the transactivation function of ESR1 mutants. (A) MCF7 cells were transfected with ERE-LUC and indicated ER expressing plasmid, both in the absence and presence of XBP1s. The LUC activity of lysate without exogenous ER and spliced XBP1 was set as 1. Normalised LUC activity is presented as mean ± SD (n = 3). (B) MCF7 cells were transfected as in A, cell lysates were analysed by western blotting using antibodies against ER and β-actin. *P < 0.05, **P < 0.01, ***P < 0.001 two-tailed unpaired t-test. Uncropped full-length pictures of Western blotting membranes presented in the Supplementary Figure 2.
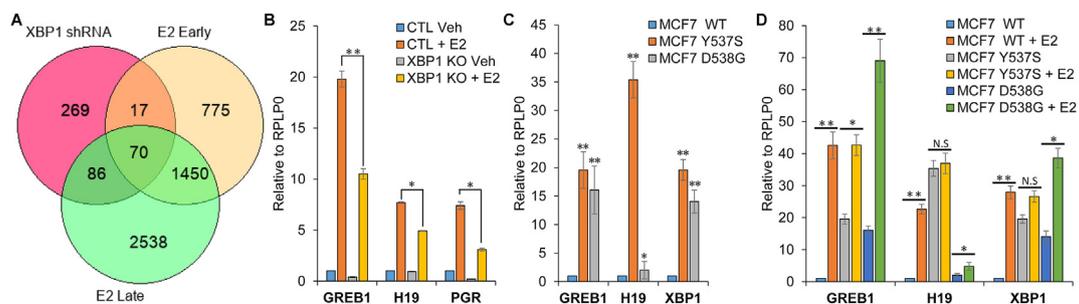
Figure 3. Estrogen-responsive genes induced by point mutants of ER require XBP1. (A) Venn diagram displaying overlapping genes between XBP1-regulated genes and estrogen- responsive genes. XBP1 shRNA: genes downregulated in XBP1 shRNA expressing cells; E2 Early: genes regulated at 4h of E2 stimulation, E2 Late; genes regulated at 24h of E2 stimulation. (B) After 2 days of synchronization in phenol red free DMEM containing 3% CSS, MCF7 control (CTL) and XBP1 knockout (XBP1 KO) cells were either treated with (Veh) or estrogen (E2) for 24h. Relative expression of the indicated genes is shown (n = 3). (C) MCF7 WT, MCF7 Y537S and MCF7 D538G cells were grown in steroid free conditions in phenol red free DMEM containing 5% CSS. Relative expression of the indicated genes is shown (n = 3). (D) After 3 days of synchronization in phenol red free DMEM containing 5% CSS, MCF7 WT, MCF7 Y537S and MCF7 D538G cells were either treated (Veh) or estrogen (E2) for 24h. Relative expression of the indicated genes is shown (n = 3). *p < 0.05, **P < 0.01, two-tailed unpaired t-test, N.S not significant.

wild-type ER (Figure 3C). In addition to the ligand-independent activity, we examined whether Y537S and D538G mutants had altered response to estrogen. We observed that MCF7 cells expressing Y537S mutant showed little further induction of H19 and XBP1 upon addition of estrogen, whereas cell expressing either wild-type or D538G mutant receptor, showed induction after estrogen stimulation (Figure 3D).

3.4. Depletion of XBP1 reduces growth of MCF7-Y537S cells and sensitizes them to endocrine therapy

To better understand the role of XBP1 in MCF7-Y537S cells, we abrogated the expression of XBP1 using lentiviral shRNAs. We observed that XBP1-targeting shRNA reduced the UPR-induced expression of

XBP1s (Figure 4A and SF3) as well as the induction of XBP1s-dependent UPR target genes (Figure 4B). Further, we observed a significant reduction in the colony formation of MCF7-Y537S XBP1-shRNA cells as compared to MCF7-Y537S control cells (Figure 4C, D). MTS assay showed that growth of MCF7-Y537S XBP1-shRNA cells was reduced as compared to MCF7-Y537S control cells (Figure 4E). There was no difference in the growth of parental MCF7 cells and MCF7-Y537S XBP1-shRNA cells. The growth of MCF7-Y537S control cells was higher than parental MCF7 cells and MCF7-Y537S XBP1-shRNA (harbouring Y537S mutation and lacking XBP1) cells behaved like parental MCF7 cells. Together, these data indicate that compromised XBP1 expression reduces the growth and proliferation of cells expressing Y537S mutant. Next, we treated MCF7-Y537S control and MCF7-Y537S XBP1-shRNA cells with

tamoxifen. To confirm the partial resistance of MCF7-Y537S control cells, parental MCF7 cells were also used. As expected, MCF7-Y537S control showed partial resistance towards tamoxifen as compared to parental MCF7 cells (Figure 4F). Further, we found increased sensitivity of MCF7-Y537S XBP1-shRNA cells as compared to MCF7-Y537S control cells (Figure 4F). Notably, MCF7-Y537S XBP1 shRNA cells were more sensitive than parental MCF7 cells (Figure 4F). These results suggest that inhibition of XBP1 could restore the sensitivity towards anti-estrogens in the MCF7 cells expressing Y537S mutant. Finally, to determine the role of XBP1 on the constitutive and E2-stimulated gene expression in cells expressing Y537S mutant, we used Y537S CTL and Y537S XBP1 shRNA cells. To confirm ligand-independent expression of estrogen-responsive genes in MCF7-Y537S CTL cells, parental MCF7 cells were also used. We observed that MCF7 cells expressing Y537S mutants showed a significant increase in the constitutive ligand-independent expression of E2-responsive genes (GREB1, and PGR) as compared to the cells expressing wild-type ER (Figure 5) which was inhibited by knockdown of XBP1. Further, we observed that MCF7 cells expressing Y537S mutant showed induction of GREB1 and PGR upon addition of estrogen, which was attenuated by knockdown of XBP1 (Figure 5). These results show that knockdown of XBP1 in MCF7-Y537S cells compromised the basal as well as E2-stimulated expression of estrogen responsive genes.

4. Discussion

Point-mutations and rearrangements in the ESR1 gene are common in metastatic, endocrine-resistant breast cancers [29, 30]. Hotspot point mutations occurring in the ligand-binding domain of ESR1 have been found in up to 40% of endocrine-resistant breast cancers [6]. The two most prevalent ESR1 mutations (Y537S and D538G) show estrogen-independent transactivation activity, and resistance to anti-estrogens [7]. Genomic rearrangement of ESR1 locus producing ESR1 fusion genes are implicated in resistance to endocrine therapy [12]. ESR1-YAP1 and ESR1-DAB2 have been shown to drive resistance to

endocrine therapy and metastasis in breast cancer [10, 11]. Both of these ESR1 fusion proteins retain the N-terminus of ESR1 coding for the DNA-binding and nuclear localization domains; show estrogen-independent transactivation activity, and resistance to anti-estrogens. Despite the emergence of several combination treatments including CDK4/6 inhibitors with the hormonal therapies, development of resistance poses a significant challenge [6].

In agreement with previous reports, we confirmed ligand-independent activity of point mutants (Y537S, D538G) and fusion mutants (ESR1-YAP1, ESR1-DAB2) of ESR1. The expression of point mutants (Y537S, D538G) was comparable to the wild-type ER which indicates that elevated ER-transactivation of point mutants (Y537S, D538G) is due to the increased function of point mutants. Indeed, point mutations (Y537S, D538G) in the ligand-binding domain of ESR1 cause structural rearrangements leading to their constitutive interaction with co-activator, NCOA3 in a ligand-independent manner [7]. NCOA3 is an oncogenic co-activator and interacts with nuclear receptors to enhance the expression of cognate target genes [31]. Unlike point mutants we observed an increased expression of fusion proteins (ESR1-YAP1 and ESR1-DAB2) as compared to wild-type ER, which can partly explain the enhanced ER-transactivation activity of these mutants. Further, we found that XBP1s synergistically increased the transactivation function of ESR1 mutants. The N- and C-terminal region of ER can physically interact with XBP1s and contribute to increased ER transcriptional activity by XBP1s [26]. Our results suggest that increased transcriptional function of point-mutants and fusion-mutants of ESR1 by XBP1s may involve different mechanisms. Co-expression of XBP1s has no observable effect on expression of wild-type and point mutants of ER but it increased the expression of (ESR1-DAB2 and ESR1-YAP1) fusion mutants. Further studies are required to determine the mechanisms responsible for increased steady-state protein levels of fusion proteins (ESR1-YAP1 and ESR1-DAB2) as compared to wild-type and point-mutants of ER.

Several studies have shown that activation of estrogen-responsive genes is a common feature of endocrine-resistant ER-positive breast
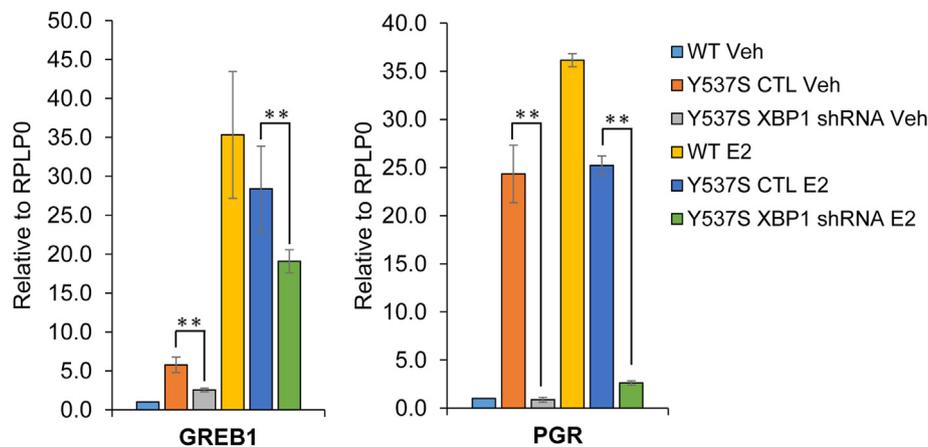
Figure 5. Depletion of XBP1 reduces the expression of estrogen-responsive genes in MCF7-Y537S cells. Indicated sub-clones of MCF7 cells were synchronized for 3 days in phenol red free DMEM containing 5% CSS. Cells were either treated with (Veh) or estrogen (E2) for 24h. Relative expression of the GREB1 and PGR was evaluated by RT-PCR. A representative of two experiments performed in triplicates is shown. *p < 0.05, **P < 0.01, two-tailed unpaired t-test.

cancers harbouring ESR1 mutations [29]. The expression of XBP1s is increased upon E2-stimulation, which in turn, enhances ER-dependent transcriptional activity [22]. We found the increased expression of XBP1 in cells harbouring Y537S and D538G mutations. Our results showed a significant overlap between XBP1-regulated genes and estrogen-responsive genes in context of ER-positive breast cancer. In addition, XBP1 was required for optimal induction of estrogen-responsive genes that were upregulated in cells harbouring Y537S and D538G mutations. Furthermore, loss of XBP1 reduced growth and sensitized the cells expressing Y537S mutants to tamoxifen. In this study we have characterised the effect of XBP1 inhibition on the growth and response to endocrine therapies in the cells expressing point mutants of ESR1. The effect of XBP1 inhibition on the growth and response to endocrine therapies in the cells expressing fusion mutant of ESR1 needs further investigation. To the best of our knowledge, this is the first report to show the functional interaction between XBP1 and gain-of-function mutants of ESR1 in breast cancer. Our results provide proof-of-concept for targeting of XBP1 in combination with anti-estrogen therapy to overcome endocrine resistance in ESR1-mutant cancers. Finally, our results suggest that targeting of XBP1 in combination with endocrine therapy may improve clinical outcomes in endocrine-resistant breast cancer patients.

Declarations

Author contribution statement

D. Barua Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

A. Gupta and S. Gupta: Conceived and designed the experiments; Wrote the paper.

B. Abbasi: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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