

ORIGINAL ARTICLE

Gene expression profiling identifies *FYN* as an important molecule in tamoxifen resistance and a predictor of early recurrence in patients treated with endocrine therapy

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To elucidate the molecular mechanisms of tamoxifen resistance in breast cancer, we performed gene array analyses and identified 366 genes with altered expression in four unique tamoxifen-resistant (TamR) cell lines vs the parental tamoxifen-sensitive MCF-7/S0.5 cell line. Most of these genes were functionally linked to cell proliferation, death and control of gene expression, and include *FYN*, *PRKCA*, *ITPR1*, *DPYD*, *DACH1*, *LYN*, *GBP1* and *PRLR*. Treatment with *FYN*-specific small interfering RNA or a SRC family kinase inhibitor reduced cell growth of TamR cell lines while exerting no significant effect on MCF-7/S0.5 cells. Moreover, overexpression of *FYN* in parental tamoxifen-sensitive MCF-7/S0.5 cells resulted in reduced sensitivity to tamoxifen treatment, whereas knockdown of *FYN* in the *FYN*-overexpressing MCF-7/S0.5 cells restored sensitivity to tamoxifen, demonstrating growth- and survival-promoting function of *FYN* in MCF-7 cells. *FYN* knockdown in TamR cells led to reduced phosphorylation of 14-3-3 and Cdc25A, suggesting that *FYN*, by activation of important cell cycle-associated proteins, may overcome the anti-proliferative effects of tamoxifen. Evaluation of the subcellular localization of *FYN* in primary breast tumors from two cohorts of endocrine-treated ER+ breast cancer patients, one with advanced disease ($N=47$) and the other with early disease ($N=76$), showed that in the former, plasma membrane-associated *FYN* expression strongly correlated with longer progression-free survival ($P < 0.0002$). Similarly, in early breast cancer patients, membrane-associated expression of *FYN* in the primary breast tumor was significantly associated with increased metastasis-free ($P < 0.04$) and overall ($P < 0.004$) survival independent of tumor size, grade or lymph node status. Our results indicate that *FYN* has an important role in tamoxifen resistance, and its subcellular localization in breast tumor cells may be an important novel biomarker of response to endocrine therapy in breast cancer.

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INTRODUCTION

More than 80% of breast cancers express the estrogen receptor (ER+) and depend on estrogen to drive tumor growth and progression. Treatment of ER+ breast cancers has primarily relied on the use of tamoxifen, a selective ER modulator (SERM) that interferes with ER function.¹ Adjuvant treatment with tamoxifen significantly reduces the risk of recurrence and death in all age groups. A meta-analysis of 21 457 women with breast cancer in 20 trials of adjuvant tamoxifen therapy showed a reduction of 15-year breast cancer mortality rates by at least a third.² More recently, aromatase inhibitors (AIs), which block estrogen synthesis, have been introduced in the clinic for postmenopausal patients.³ Despite overall superiority of the AIs, tamoxifen is still the recommended treatment modality for premenopausal breast cancer patients and patients with contraindications for treatment with AIs. In addition, the side effects of the drugs differ and some patients may not be eligible for a given drug owing to comorbidities.⁴ It is therefore rational to maintain tamoxifen as an adjuvant treatment option.

Resistance to tamoxifen treatment is a major clinical problem as a proportion of ER+ patients do not respond to therapy from the start or acquire resistance at a later stage.⁵ As a consequence,

recurrence occurs in ~30% of patients after 15 years of follow-up despite adequate treatment.² A large body of evidence suggests that complex interactions between the ER and growth factor signaling pathways have a role in the development of endocrine resistance.⁵ Indeed, non-genomic actions of ER are known to include interaction with, and activation of, signaling kinases such as epidermal growth factor receptor, human epidermal growth factor receptor 2, insulin growth factor 1 receptor, SRC kinase, phosphatidylinositol 3-kinase and mitogen-activated protein kinase.^{6–8} Studies on tamoxifen resistance using breast cancer cell lines showed elevated levels of ER α in the cytoplasm and plasma membrane.⁹ The epidermal growth factor receptor family, including epidermal growth factor receptor and human epidermal growth factor receptor 2, has been implicated in the enhanced activation of PI3K/AKT, mitogen-activated protein kinase, protein kinase C (PKC) and SRC.^{10–13} On the other hand, ER can be activated by a ligand-independent mechanism as a consequence of phosphorylation in the AF-1 domain following cross talk with growth factor receptor signaling.^{8,14} Increased nuclear factor-kappaB signaling has been shown to influence the response to tamoxifen treatment.^{15,16} However, to date, only ER is approved as predictive marker for response to endocrine therapy.

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New predictive markers for response to endocrine therapy are urgently required to improve treatment outcomes.

Measurement of global gene expression profiles that reflect the status of ER signaling and related pathways could elucidate the molecular characteristics of breast cancers and help identify predictive markers of endocrine treatment responses. In this study, we utilized a panel of well-characterized, clinically relevant tamoxifen-resistant (TamR) cell lines generated from an MCF-7 breast cancer cell line^{17–19} and performed genome-wide gene expression analysis to identify genes that exhibit altered expression in TamR vs parental tamoxifen-sensitive breast cancer cells. The resistant cell lines have reduced ER expression and lost PGR expression,^{18,19} which is also observed in relapsing ER+ breast cancer patients.²⁰ Functional analysis of the top candidate genes overexpressed in resistant cell lines identified *FYN* to be essential for the survival and growth of TamR cell lines and a useful biomarker to identify patients receiving adjuvant tamoxifen monotherapy at risk of developing metastatic disease.

RESULTS

Altered gene expression profile associated with tamoxifen resistance in MCF-7 breast cancer cell lines as determined by gene array analysis

Genome-wide analysis of gene expression using gene array was performed on four TamR cell lines, TamR1, TamR4, TamR7 and

TamR8, and compared with the tamoxifen-sensitive parental breast cancer cell line MCF-7/S0.5. Two to three samples of each of the TamR cell lines and seven samples of the parental cell line were arrayed. Evaluation of mean variance between the replicates of the same cell lines and between the TamR and parental cell lines showed mean F ratios of 4.8 and 26.8, respectively, demonstrating significantly larger gene expression variance between the TamR and parental cell lines than that between individual samples of a given cell line. A total of 366 genes were identified as exhibiting significantly altered expression (\geq twofold alteration in expression and false-discovery rate < 0.05) in TamR vs MCF-7/S0.5 cell lines. Among these, 256 were up regulated, whereas the remaining genes were downregulated in TamR cell lines. A heat map of differentially expressed genes is shown in Figure 1a and the genes are listed in Supplementary Table S1. Signaling pathways altered in association with tamoxifen resistance in this model and known to be involved in growth regulation are listed in Supplementary Table S2, including prolactin, PKA, extracellular signal-regulated kinase/mitogen-activated protein kinase, cyclin-dependent kinase, growth hormone and breast cancer regulation by stathmin 1 signaling pathways.

Analysis of genes commonly exhibiting altered expression using the Ingenuity Pathway Analysis software (IPA) revealed *FYN*, *PRKCA*, *ITPR1*, *DPYD*, *DACH1*, *GBP1*, *LYN* and *PRLR* to be central in networks with primary functions relevant to endocrine resistance,

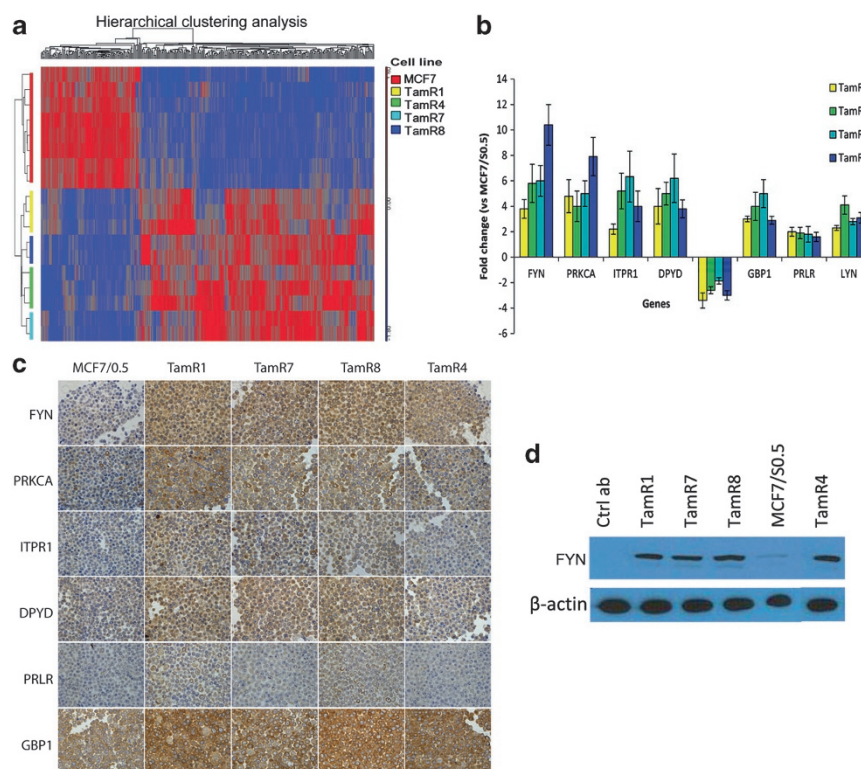


Figure 1. Genes and proteins exhibiting altered expression in TamR vs tamoxifen-sensitive parental (MCF-7/S0.5) breast cancer cells. **(a)** Hierarchical clustering of TamR cells and MCF-7/S0.5 was performed using Pearson's correlation. Raw Affymetrix intensity values were background corrected and normalized using Robust Multiarray Average. One-way analysis of variance was performed and probes with \geq twofold change in expression and a false-discovery rate of < 0.05 were used to select 366 genes exhibiting altered expression in TamR vs MCF-7/S0.5 cells. The cell lines are presented vertically and genes are clustered horizontally. Red (higher) and blue (lower) expression in TamR vs MCF-7/S0.5. **(b)** Verification of the altered gene expression observed in the gene array for selected genes including, *FYN*, *PRKCA*, *ITPR1*, *DPYD*, *DACH1*, *GBP1*, *PRLR* and *LYN* using quantitative RT-PCR. Four different TamR cells (TamR1, TamR7, TamR8 and TamR4) and the parental (MCF-7/S0.5) were analyzed, expression was normalized using the *PUM1* gene and depicted as the relative expression in TamR vs MCF-7/S0.5 cells. One-way analysis of variance followed by Turkey's multiple comparison tests showed that the levels of expression of all eight genes in TamR cells differed significantly from MCF-7/S0.5 cells. **(c)** Protein expression patterns of selected genes determined by immunocytochemistry. TamR and MCF-7/S0.5 cells were formalin-fixed and embedded in paraffin, and sections were stained for FYN, PKCA, ITPR1, DPYD, PRLR and GBP1. Magnification $\times 40$. **(d)** Western blot analysis of FYN expression in MCF-7/S0.5 and TamR cell lines. β -Actin was used as loading control.

including involvement in proliferation, cell death and cell-to-cell signaling. Furthermore, 77 of the 366 differentially expressed genes were estrogen regulated, as listed in the estrogen-regulated gene database (ERGDB)²¹ (Supplementary Table S3), demonstrating a significant overlap ($P=2.56 \times 10^{-10}$) and indicating that estrogen-regulated pathways are significantly altered in TamR cell lines.

To confirm the altered expression of selected genes identified by the gene array, *FYN*, *PRKCA*, *ITPR1*, *DPYD*, *DACH1*, *GBP1*, *LYN* and *PRLR* expression were evaluated in TamR vs MCF-7/S0.5 using quantitative RT-PCR. All eight genes exhibited expression patterns similar to those observed by gene array analysis (Figure 1b).

TamR cell lines express higher protein levels of FYN, PKC α , ITPR1 and DPYD than the parental cell line

To investigate whether the differences in expression observed at the mRNA level were also reflected at the protein level, immunocytochemical analysis was performed and it showed higher expression of FYN, PKCA, ITPR1 and DPYD proteins in TamR vs MCF-7/S0.5 (Figure 1c). GBP1 was excluded from further functional analysis because the gene was highly expressed at the protein level in all cell lines (Figure 1c). Comparable protein expression levels were observed for both PRLR and GBP1. FYN, PKCA, ITPR1, DPYD and GBP1 expression were found in the cytoplasm and cell membrane, but not in the nucleus. FYN expression was also tested by western blot analysis and showed higher expression in all TamR cell lines, whereas the protein was barely detectable in MCF-7/S0.5 (Figure 1d).

Knockdown of *FYN* in TamR breast cancer cell lines impairs cell growth

To evaluate whether increased expression of the *FYN*, *PRKCA*, *ITPR1*, *PRLR*, *DPYD* and *LYN* genes is important for the growth and survival of TamR cells, the genes were knocked down by small interfering RNA (siRNA) leading to over 70% reduction in expression as determined by quantitative RT-PCR (Figure 2a). Knockdown of *FYN* resulted in significantly reduced growth of TamR1 cells ($P < 0.05$), whereas the effect of *PRKCA* knockdown did not reach statistical significance ($P=0.058$) and *ITPR1*, *PRLR*, *DPYD* and *LYN* knockdown showed no significant effect on cell growth (Figure 2b), indicating that upregulation of *FYN* may be important for the resistance phenotype.

We next performed *FYN* knockdown in additional TamR cell lines using two different siRNAs and the mixture. This resulted in up to 90% reduction in *FYN* mRNA levels in both the parental cell line and the four TamR cell lines (Figure 3a) and a corresponding decrease in FYN protein expression as evaluated by western blotting (Figure 3b). Using a colorimetric assay, knockdown of *FYN* was associated with significant reduction in cell growth in all TamR cell lines, whereas no significant effect was observed on the parental cell line (Figures 3c and d). The effect of *FYN* knockdown was comparable in the presence or absence of tamoxifen, suggesting that the importance of FYN to the growth and survival of TamR cells is independent of the ER pathway. The reduced cell proliferation following *FYN* knockdown seen in the colorimetric assay was confirmed using a BrdU incorporation assay (Supplementary Figure S1) in which DNA synthesis was significantly reduced following *FYN* knockdown. Moreover, to evaluate the possible effect of *FYN* knockdown on cell death, we measured the apoptosis level using Cell Death Detection ELISA^{plus} (Roche, Hvidovre, Denmark), which showed a small, but not significant, increase in apoptosis compared to cells transfected with scrambled siRNA control in four of the cell lines (Supplementary Figure S2).

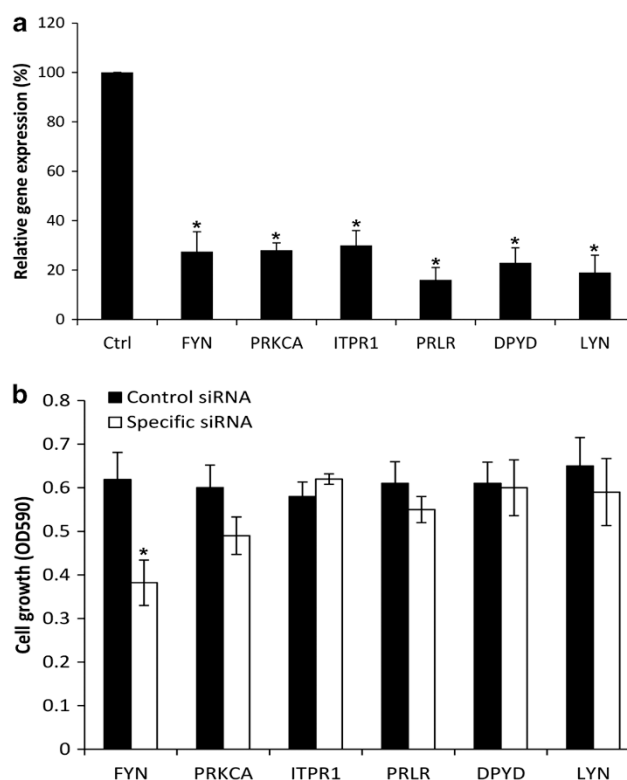


Figure 2. Knockdown of selected genes identified to exhibit higher expression in TamR vs MCF-7/S0.5 and the effect on cell growth. (a) TamR1 cells were transfected with siRNAs specific for *FYN*, *PRKCA*, *ITPR1*, *PRLR*, *DPYD* or *LYN* leading to a statistically significant ($*P < 0.01$) reduction in gene expression compared with TamR1 cells transfected with control siRNA. (b) Knockdown of *FYN* ($P=0.042$) and *PRKCA* ($P=0.058$) with specific siRNA vs negative control siRNA decreased cell growth in TamR1 in the presence of 10^{-6} M tamoxifen, whereas knockdown of *ITPR1*, *PRLR*, *DPYD* or *LYN* did not alter cell growth ($*P < 0.05$).

Overexpression of FYN in tamoxifen-sensitive MCF-7/S0.5 cells reduces tamoxifen responsiveness

To further evaluate the importance of FYN overexpression on tamoxifen responsiveness, tamoxifen-sensitive parental MCF-7/S0.5 cells were separately transfected with an expression plasmid carrying either of two *FYN* transcript variants (variants 1 or 2). Both transcript variants resulted in a marked increase in FYN protein expression (Figure 4a). FYN overexpression in MCF-7/S0.5 cells in the absence of tamoxifen did not significantly affect their growth; however, in the presence of tamoxifen, FYN overexpression resulted in reduced tamoxifen sensitivity compared with MCF-7/S0.5 cells transfected with empty vector, indicating that FYN mediates growth and survival signals that abrogate the growth inhibitory effect of tamoxifen (Figure 4b). Next, we performed *FYN* knockdown in the FYN-overexpressing MCF-7/S0.5 cells using siRNA transfection and found marked reduction of FYN expression (Figure 4c) and restored sensitivity to tamoxifen (Figure 4d). This result further supports the contention that increased expression of *FYN* is an important factor in overcoming the growth inhibitory effect of tamoxifen.

SRC family kinase inhibitor PP2 impairs the growth of TamR breast cancer cell lines

On the basis of the observation that knockdown of FYN reduced the growth of TamR cell lines, we examined whether PP2, a specific chemical inhibitor of SRC family kinases (SFK), could inhibit growth of TamR and parental MCF-7/S0.5 cell lines.

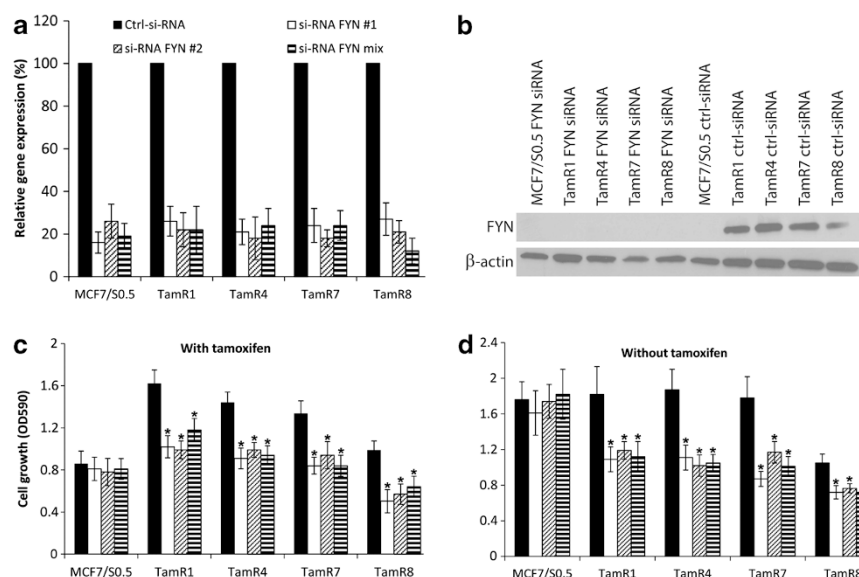


Figure 3. siRNA-mediated *FYN* knockdown and its effect on growth of the parental and four TamR cell lines in the presence or absence of tamoxifen. Transfection of the four TamR (TamR1, TamR7, TamR8 and TamR4) and the parental MCF-7/S0.5 cell lines resulted in significant reduction of *FYN* expression at both the mRNA (a) and protein (b) levels. mRNA and protein expression levels were evaluated 48 and 72 h following transfection, respectively. Knockdown of *FYN* resulted in reduced proliferative capacity of TamR cells, but not of MCF-7/S0.5 cells in medium with (c) or without (d) 10⁻⁶ M tamoxifen as measured 72 h following siRNA transfection using a crystal violet-based colorimetric assay. Two separate siRNAs (si-FYN #1, si-FYN #2 and their mixture) were used in the experiments shown in a, c and d. Lysates from cells transfected with the mix of two siRNAs were used in the western blot analysis shown in b. Representative of three separate experiments each performed in triplicate are shown. Note that MCF-7/S0.5 cells showed $\geq 40\%$ less growth in medium with tamoxifen (c) compared with medium without tamoxifen (d) (* $P < 0.05$).

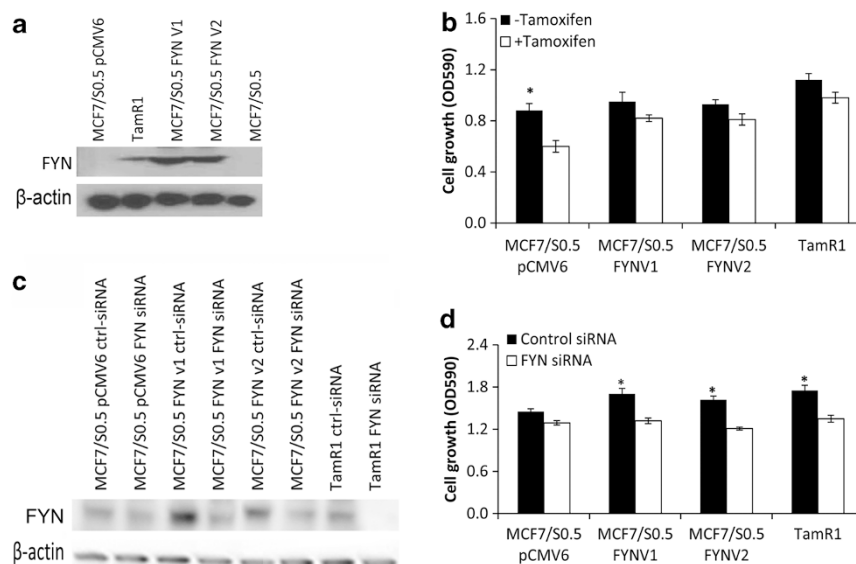


Figure 4. Overexpression of *FYN* in MCF-7/S0.5 cells reduces tamoxifen responsiveness. (a) *FYN* western blot analysis was performed with proteins extracted from tamoxifen-sensitive MCF-7/S0.5 cells transfected with mammalian expression vector carrying DNA coding for *FYN* variant 1 (MCF-7/S0.5 FYN v1) or variant 2 (MCF-7/S0.5 FYN V2) and cells transfected with empty vector (MCF-7/S0.5 pCMV6). Lysates from untransfected TamR1 cells and MCF-7/S0.5 cells were also included. (b) The growth of MCF-7/S0.5 pCMV6, MCF-7/S0.5 FYN v1, MCF-7/S0.5 FYN V2 and TamR1 cells in the presence or absence of 10⁻⁶ M tamoxifen was determined after 72 h by a colorimetric assay. (c) *FYN* gene knockdown was performed on MCF-7/S0.5 pCMV6, MCF-7/S0.5 FYN V1, MCF-7/S0.5 FYN V2 and TamR1 cells and western analysis was performed to evaluate the expression of *FYN*. (d) Growth of cells transfected with siRNA specific to *FYN* or negative control siRNA was evaluated using a colorimetric assay. * $P < 0.05$. A representative of three independent experiments, each performed in triplicate, is shown.

Treatment with 2.4 μ M PP2 had no effect on growth of the parental cell line and combined treatment with tamoxifen and PP2 did not result in increased growth inhibition compared with tamoxifen alone. As expected, the TamR cell lines were not growth inhibited by tamoxifen, whereas PP2 exerted significant growth inhibition

on all TamR cell lines and the effect was similar in the presence or absence of tamoxifen, indicating no cross talk between FYN- and ER-mediated signaling (Figure 5a). Higher concentration of PP2 ($\geq 5 \mu$ M) dramatically impaired the growth of all the cell lines tested, including the MCF-7/S0.5 (result not shown).

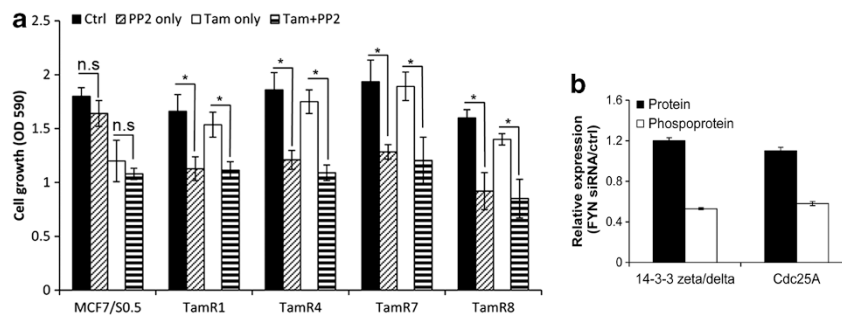


Figure 5. Selective SKF inhibitor PP2 inhibits growth of TamR cell lines. **(a)** Growth of MCF-7/S0.5 and TamR cell lines following treatment with PP2 (2.4 μ M) or its solvent, ethanol (Ctrl), in medium with or without tamoxifen. Cell growth was determined at 72 h using a colorimetric assay. A representative of three independent experiments, each performed in triplicates, is shown. * $P < 0.01$, not significant (n.s.) **(b)** Relative expression of 14-3-3 zeta/delta and Cdc25A and their phosphorylated forms following FYN siRNA or control siRNA knockdown in four different TamR cell lines. The mean of the intensity ratio of FYN siRNA /control siRNA for the four TamR cell lines is shown.

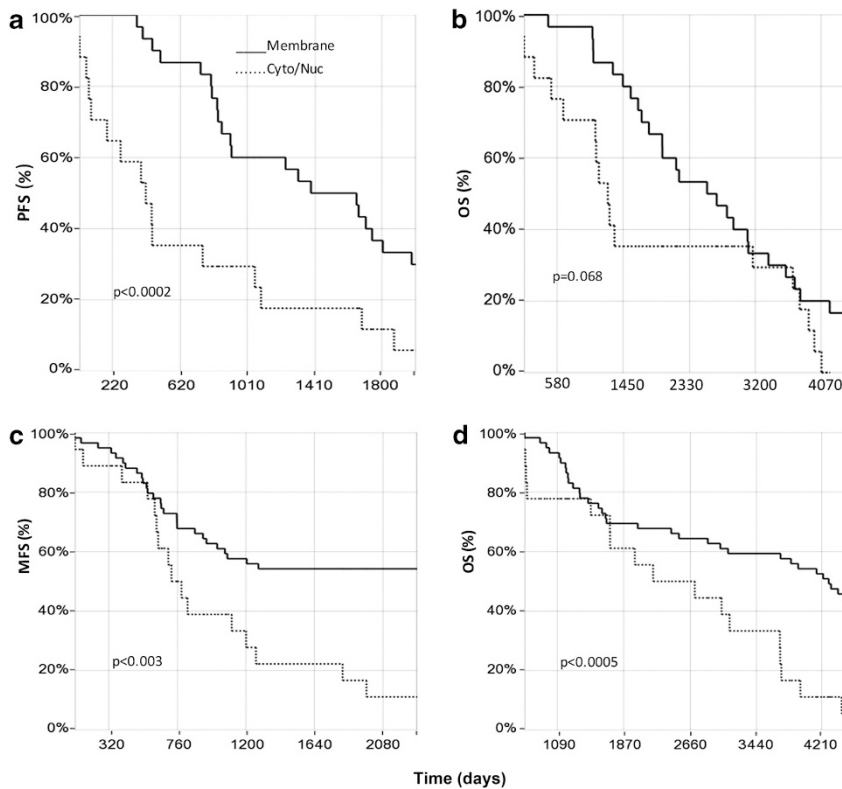


Figure 6. Survival curves generated by Kaplan–Meier estimates relative to subcellular FYN expression in primary breast tumors. Kaplan–Meier plots evaluating **(a)** progression-free survival (PFS) and **(b)** overall survival (OS) according to subcellular localization of FYN protein in ER+ primary breast tumors from patients with advanced disease treated with tamoxifen or letrozole. **(c)** Metastasis free survival (MFS) expressed as time to metastasis and **(d)** OS according to subcellular localization of FYN protein in primary breast tumors from ER+ early breast cancer patients treated with 5 years of adjuvant tamoxifen monotherapy. A two-sided P -value was calculated using log-rank testing.

siRNA-mediated knockdown of *FYN* reduces phosphorylation of 14-3-3 zeta/delta and Cdc25A

To understand how *FYN* expression may influence tamoxifen resistance, the four TamR cells before and following *FYN* knockdown were analyzed for expression of downstream cell cycle-associated molecules using protein/phosphoprotein arrays. Phosphorylation of 14-3-3 zeta/delta (Thr232) and Cdc25A (Ser124), both key regulators of cell cycle progression, were consistently reduced in all four TamR cell lines following *FYN* knockdown (Figure 5b), but not in the parental cell line (data not shown), suggesting that *FYN*, by activation of important cell cycle-associated proteins, may overcome the anti-proliferative effects of

tamoxifen. This is further supported by the observation that the SRC target breast cancer anti-estrogen resistance 1 (BCAR1), which has been described to promote anti-estrogen-resistant cell growth,²² showed higher phosphorylation levels in TamR cell lines compared with MCF-7/S0.5 cells (Supplementary Figure S3).

Subcellular localization of *FYN* in ER+ primary breast tumors strongly correlates with clinical outcome

To elucidate the clinical relevance of our findings in the cell line model suggesting *FYN* is a key component in the survival and growth of TamR cells, we evaluated *FYN* expression in ER+

primary breast tumors from two cohorts of patients using immunohistochemistry. One cohort was comprised of patients with advanced ER+ breast cancers who had received tamoxifen or letrozole monotherapy ($N=47$) and the other was comprised of postmenopausal patients with early ER+ breast cancers ($N=76$) treated with adjuvant tamoxifen for 5 years. Clinical and pathological characteristics of the two patient cohorts are listed in Supplementary Table S4. All tumors from patients with advanced disease and 73 of the 76 (96%) tumors from early breast cancer patients showed detectable FYN expression (Supplementary Table S5).

Evaluation of the subcellular localization of FYN in the advanced disease cohort showed that 63.8% (30/47) of the tumors exhibited FYN staining simultaneously in the plasma membrane and cytoplasm, whereas 36.2% (17/47) exhibited FYN staining exclusively in the cytoplasm (Supplementary Table S5). The intensity of FYN and the primary clinical endpoints showed no significant correlation. However, in both cohorts, the subcellular location of FYN strongly correlated with the clinical outcome. Advanced breast cancer patients with tumors exhibiting plasma membrane-associated FYN showed significantly longer progression-free survival than those with cytoplasmic and no detectable plasma membrane-associated FYN expression (median time to progression of 54.4 vs 14.9 months, $P < 0.0002$; Figure 6a). The correlation of FYN subcellular localization and overall survival (OS) did not reach statistical significance ($P=0.068$; Figure 6b). Subgrouping the patients based on the endocrine treatment (tamoxifen or letrozole) showed similar results where plasma membrane-associated FYN expression significantly correlated with longer time to progression for both the tamoxifen- ($P < 0.004$) and letrozole ($P < 0.004$)-treated populations (Supplementary Figure S4), but the correlation with OS did not reach statistical significance in the subgroups.

In the ER+ early breast cancer patients treated with tamoxifen, 73.7% (56/76) of the tumors exhibited FYN staining simultaneously in the plasma membrane and cytoplasm, 18.4% (14/76) exhibited FYN staining exclusively in the cytoplasm, 3.9% (3/76) exhibited FYN staining in the nucleus, whereas 3.9% (3/76) exhibited no detectable FYN in the tumor cells (Supplementary Table S5). Similar to the advanced disease cohort, plasma membrane-associated FYN expression was significantly associated with longer time to metastasis (85.0 vs 24.9 months, $P < 0.003$) and longer OS (median survival time of 142.1 vs 80.0 months, $P < 0.0005$), as illustrated in the Kaplan–Meier plots (Figures 6c and d), compared with those with exclusively cytoplasmic or nuclear FYN. In this cohort, like in the first, no correlation between the overall intensity of FYN expression and clinical end points was observed (data not shown). Representative breast cancer sections showing membrane and cytoplasmic FYN staining, respectively, can be seen in Supplementary Figure S5.

Cox proportional hazard regression analysis of metastasis-free survival (MFS) according to subcellular localization of FYN and clinicopathological characteristics including tumor size, node status and tumor grade (Table 1) showed that the risk of recurrence was significantly decreased in patients with tumors expressing plasma membrane-associated FYN or no FYN expression (hazard ratio (HR) 0.46, $P=0.036$) in the presence of < 4 positive lymph nodes (HR 0.49; $P < 0.002$), whereas the effect of low tumor grade did not reach statistical significance (HR 0.57; $P=0.15$). The corresponding Cox analysis of OS showed that plasma membrane-associated FYN or undetectable FYN expression (HR 0.32; $P=0.0006$), < 4 positive lymph nodes (HR 0.38; $P=0.004$) and low tumor grade (HR 0.32; $P=0.015$) were identified as independent prognostic factors. Tumor size was not significantly correlated to time to metastasis or death in this cohort.

Table 1. Cox multivariate regression analysis of selected risk factors in the cohort of patients with ER+ early breast cancer treated with tamoxifen monotherapy

Predictor	Hazard ratio		P-values	
	Metastasis	Death	Metastasis	Death
Plasma membrane FYN	0.56	0.38	< 0.04	0.004
< 4 Positive lymph nodes	0.49	0.38	< 0.007	0.004
Low malignancy grade	0.57	0.29	0.15	0.007

DISCUSSION

Several studies have identified proteins and pathways that seem to have important roles in endocrine resistance,^{23–29} but thus far, human epidermal growth factor receptor 2 overexpression is the only biomarker in clinical use for selection of ER+ patients with poor response to endocrine therapy.³⁰ In this study, using a robust global gene expression analysis, we identified molecules associated with tamoxifen resistance in an established clinically relevant breast cancer cell line model. Among the 366 genes exhibiting altered expression, several were previously reported to be associated with endocrine resistance, including *IGF1R*,^{25,31,32} *PRKD1*³³ and *ITGA6*,³⁴ supporting the validity of the data set. Of the previously uncharacterized genes associated with endocrine resistance, we focused on the kinase FYN, which exhibited increased expression in all four TamR. FYN reduction using specific siRNA resulted in significant growth inhibition of TamR cell lines independent of tamoxifen, but not in the tamoxifen-sensitive parental cell line. Our experiments further showed decreased tamoxifen sensitivity upon overexpression of FYN in MCF-7/S0.5 cells, clearly demonstrating that FYN has survival- and/or growth-promoting activity in the MCF-7/S0.5 cells. It is well known that introduction of growth-promoting genes such as epidermal growth factor receptor and human epidermal growth factor receptor 2 reduces the sensitivity of MCF-7 cells to tamoxifen treatment.^{35,36} The finding that FYN was overexpressed in all the four TamR cell lines and that abrogation of FYN function by either siRNA-mediated knockdown or treatment with the FYN-targeting kinase inhibitor PP2 resulted in significant growth inhibition in all TamR cell lines indicated a causal role for FYN in TamR cell growth. The inhibitory effect of FYN knockdown and of treatment with PP2 was similar in both the presence and absence of tamoxifen, suggesting that FYN acts independently of ER. Although we have not proven that the PP2-mediated growth inhibition of TamR cells was due to inhibition of FYN kinase activity, as the drug targets multiple SFKs at high concentrations, it seems highly plausible as FYN and LYN were the only SFKs that exhibited increased expression, and knockdown of LYN did not affect cell growth.

Among the genes that exhibited altered expression in TamR vs parental cell lines in the gene array analysis, 77 were regulated by ER, including *ABCG1*, *CSTSO*, *CXCL12*, *GREB1*, *JDP2*, *PGR*, and several of the *DUSP* family genes, which may be explained by the reduced ER level in all the TamR cell lines.³⁷ Although the ER level was reduced, growth of the TamR cell lines was at least partially promoted via the ER, but ER-independent growth-promoting pathways were also involved in the growth of TamR cells.³⁷ Our data herein suggest that FYN may be an important contributor to the ER-independent growth promotion of TamR cell lines.

The involvement of members of SFKs, including *SRC*, *FYN*, *YES*, *HCK*, *LYN*, *BLK*, *LCK* and *YRK*,³⁸ in cancer pathogenesis and resistance to anticancer agents has been frequently reported.^{11,39–41} SFKs were among the first oncogenes recognized in cancer biology and have remained to be of great interest because of their role in mediating extracellular stimuli to the nucleus. Several *in vitro* studies have suggested a role of *SRC* in

acquired resistance to endocrine drugs where its increased expression reportedly resulted in increased motility and invasive phenotype of a TamR breast cancer cell line,¹¹ as well as facilitating cross talk between ER and growth factors to drive the growth of breast cancer cells.⁴² However, little is known about the role of FYN in breast cancer pathogenesis or resistance to endocrine treatment.

Some reports have shown enhanced expression of FYN in various cancer types, including glioblastoma multiforme, squamous cell carcinoma, melanoma and prostate cancer.⁴³ However, the functional consequence of the enhanced expression of FYN has not been studied. To understand how FYN expression influences resistance to tamoxifen, we evaluated the downstream signaling from FYN. Silencing of *FYN* in all four TamR cell lines resulted in downregulation of phosphorylated 14-3-3 zeta/delta and Cdc25A. Elevated levels of 14-3-3 in ER+ primary breast tumors have been reported to correlate with poor clinical outcome.⁴⁴ Cdc25A is a member of dual-specificity phosphatases, an important molecule involved in cell cycle control, which, when inactivated, contributes to cell cycle arrest.⁴⁵ Overexpression of Cdc25A has been observed in various cancer types and correlated with poor prognosis.⁴⁶

Following our finding of the importance of FYN expression in tamoxifen resistance *in vitro*, we investigated the expression of FYN protein in ER+ breast cancers from two cohorts of endocrine-treated patients using immunohistochemistry. The subcellular location of FYN significantly correlated to clinical outcome where the presence of FYN protein in the plasma membrane strongly correlated with longer MFS and OS in ER+ early breast cancer patients and longer progression-free survival in advanced disease patients, suggesting that the subcellular localization of FYN could be an important predictor of response to endocrine therapy. FYN and several other SFKs are localized in the intracellular side of the plasma membranes of cells, and their presence in other subcellular localizations has not been widely investigated. Campbell *et al.*⁴¹ reported the localization of activated SRC in the nucleus of breast cancer cells. The presence of SFKs in the cytoplasm or nucleus, but not in the membranes, indicates that the proteins may exist in a non-myristoylated form, as myristoylation has been suggested to be required for its attachment to plasma membrane⁴⁷ and to act as a regulatory mechanism controlling translocation of SFKs from the plasma membrane. A strong association between high levels of cytoplasmic SRC expression and reduced survival time following endocrine therapy in breast cancer has been reported.^{40,48} Thus, it is tempting to speculate that cytoplasmic and/or nuclear FYN may actively interact more readily with downstream molecules in ER-independent pathways to overcome the effect of tamoxifen treatment.

In conclusion, our study showed significantly increased expression of FYN in TamR cell lines, and a causal role for FYN in TamR cell growth. In primary breast tumors with poor endocrine treatment outcome, subcellular localization of FYN strongly correlated with clinical outcome in patients treated with endocrine therapy both in the early and advanced disease stages, suggesting the potential of this molecule as a biomarker. Further studies are warranted to clarify whether FYN can serve as a predictive marker of endocrine resistance by examining tumor material from cohorts comprising both treatment-naïve and endocrine-treated ER+ breast cancer patients.

MATERIALS AND METHODS

Cell lines and standard culture conditions

The human breast cancer cell line MCF-7 was originally obtained from The Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Edinburgh, UK). The MCF-7 cells were gradually adapted to grow in low serum concentration and the tamoxifen-sensitive subline MCF-7/S0.5 was

used to establish the four TamR cell lines: MCF-7/TAM^R-1 (TamR1), MCF-7/TAM^R-4 (TamR4), MCF-7/TAM^R-7 (TamR7) and MCF-7/TAM^R-8 (TamR8) by long-term treatment with 10⁻⁶ M tamoxifen as previously described.^{17,18,37,49} Cells were kept within 10 passages throughout the experiments to reduce variability between experimental results. Tamoxifen dose-response curves for the cell lines listed above have been previously published,^{17,37} showing significant growth inhibition in the range of 10⁻⁸ to 10⁻⁶ M.

Global gene expression analysis

Total RNA was purified from each of the five cell lines: TamR1, TamR4, TamR7 and TamR8 and MCF-7/S0.5. Two to three independent cultures were used for RNA purifications for each of the TamR cell line and each sample was separately analyzed on Affymetrix Gene Chip Human Genome U133 plus 2 arrays (High Wycombe, UK) according to manufacturer's guidelines. For MCF-7/S0.5 cells, RNAs from seven independent cultures were purified and arrayed separately. All data analyses were performed using Partek Genomic Suite (Partek, Inc., Chesterfield, MO, USA). Raw Affymetrix intensity measurements were background corrected, normalized and summarized into gene expression measurements using Robust Multiarray Average. Ingenuity Pathway Analysis (IPA) 8.6 (Ingenuity Systems, Redwood City, CA, USA) was used to evaluate whether the genes differentially expressed in association with tamoxifen resistance were part of an integrated and interconnected biological networks of functionally related genes. Differentially expressed gene identifiers were uploaded into IPA to map and generate putative networks based on a curated knowledge database of pathway interactions extracted from the literature. Genes that appeared to be central in relevant networks were regarded as top candidates for further functional tests.

Gene knockdown studies

siRNA targeting the specific genes and scrambled siRNA controls were synthesized by Qiagen (Copenhagen, Denmark). Cell transfection was performed using Ingenio electroporation kit according to manufacturer's instructions (Mirus, Madison, WI, USA). Transfected cells were harvested 48 or 72 h later and analyzed using real time PCR and/or western blotting. For initial evaluation of the effect on cell growth, gene knockdown was performed using a mixture of two different siRNAs and subsequently validated using the two siRNAs tested separately.

FYN overexpression

MCF-7/S0.5 cells (5 × 10⁴) were seeded in 24-well plates and incubated with 0.5 µg True ORF mammalian expression vector containing *FYN* gene variant 1 or 2 (Origene, Rockville, MD, USA) in 50 µl Opti-MEM I (Gibco, Paisley, UK) and 1.5 µl Turbofectin (Origene) for 24 h. Cells were washed with phosphate-buffered saline, trypsinized and diluted 1:10 in fresh medium containing 400 µg/ml Genitocin (Life Technologies, Naerum, Denmark) for 1 week before experiments.

Quantitative RT-PCR

Total RNA was purified using Trizol, and RevertAid Premium Reverse Transcriptase kit (Fermentas, Slangerup, Denmark) was used for cDNA synthesis. Relative quantification of gene expression was performed using SYBR green PCR Mastermix (Applied Biosystems, Naerum, Denmark). All primers were purchased from Qiagen and Pumilio homolog-1 (*PUM1*) gene was used for normalization.

Cell growth assays

siRNA-transfected cells were seeded in 24-well plates (Sigma, St Louis, MO, USA) and growth measurements were conducted after 4 days following siRNA transfection in triplicates using the crystal violet-based colorimetric assay.⁵⁰ For experiments with the specific SRC family kinase inhibitor PP2 (Tocris, Abingdon, UK), 10⁴ cells were seeded in a medium containing 2.4 µM PP2 and cell growth was determined after 3 days. Growth assays following *FYN* knockdown were confirmed using BrdU cell proliferation kit (Cell Signaling Technology, Leiden, Holland) according to the manufacturer's instructions.

Measurement of cell death

The effect of FYN knockdown on apoptotic cell death was assessed using cell death detection ELISA^{plus} (Roche, Hvidovre, Denmark) according to the manufacturer's instructions. Briefly cells transfected by FYN siRNA or negative control siRNA were seeded in 96-well plates at a density of 10^4 cells per well in the presence or absence of 10^{-6} M tamoxifen. Four days later, supernatants were removed and adherent cells were lysed in 200 μ l lysis buffer and cell lysates were assayed for levels of mono- or polynucleosomes using cell death detection ELISA^{plus} kit (Roche).

Protein/phosphoprotein antibody array

The Cell Cycle Control/DNA Damage Phospho Antibody Array kit (Fullmoon Biosystems, Sunnyvale, CA, USA) containing 238 highly specific antibodies detecting cell cycle control and DNA damage. Following FYN-specific siRNA or scrambled siRNA transfection crude cell extracts were used in the array according to manufacturer's instructions. The slides were scanned using array scanner Gene Pix 4000B (Axon Instruments, Weatherford, TX, USA). Data were normalized by dividing the average intensity signal of replicate spots with the mean intensity of all antibodies on the array. Fold changes were determined using normalized intensity signals.

Immunohistochemistry and western blotting

The generation of cell line arrays, immunohistochemistry and Western blotting were performed as previously described.⁵¹ The following primary antibodies were used: anti-FYN, anti-ITPR1, anti-PKCa, anti-GBP1 (all from Abcam, Cambridge, UK), anti-DPYD and anti-PRLR (both from Santa Cruz, Hiedelberg, Germany). Isotype-matched mouse IgG was included as negative controls. All clinical samples were coded to maintain patient confidentiality and the studies were approved by the Ethics Committee of the Region of Southern Denmark and Copenhagen and Frederiksberg Counties (approval no 01025-KF12-138-99).

Clinical samples

Formalin-fixed paraffin-embedded (FFPE) ER+ primary breast cancer samples from two cohorts of patients were analyzed. The first cohort was part of the P025 multicenter, double blind, randomized phase III clinical trial comparing letrozole with tamoxifen as first line treatment in advanced breast cancer.⁵² This cohort consisted of women with histologically or cytologically confirmed breast cancer with local (stage IIIB by American Joint Committee on Cancer Criteria, 1992) or loco-regionally recurrent disease not amenable to treatment by surgery or radiotherapy, or with metastatic disease. This population initially consisted of 88 samples from a previous study,⁵³ but tissues were only available from 47 samples, of which 25 received tamoxifen and 22 received letrozole treatment. The second cohort was comprised of 76 early breast cancer patient tissue blocks collected from the archives at the Department of Pathology at Herlev and Roskilde Hospitals, Denmark. These patients were part of a nationwide study of 1115 Danish postmenopausal early ER+ breast cancer patients receiving tamoxifen for 5 years from 1995 to 2006. All tumors were ER+ defined as >10% positively stained tumor cells. None of the early breast cancer patients had received any adjuvant cytotoxic therapy or prior endocrine treatment. The biomarker study was conducted according to the REMARK recommendations.⁵⁴

Statistical analysis

One-way analysis of variance was performed to select genes differentially expressed between the resistant vs parental MCF-7/S0.5 cell line based on \geq twofold change in expression and a false-discovery rate of < 0.05 . Hierarchical clustering using average linkage with Pearson's correlation was applied to the target genes to compare the cell lines. Mean F ratios as determined by Partek Genomic Suite were used to assess the intra and intergroup variability between the cell lines. A one tailed t-test was used to compare groups in growth assays. Associations between cellular localization of FYN and other clinicopathological characteristics were analyzed by Fischer's exact test. P-values are two-tailed. Time to progression, also called progression-free survival, was defined as the interval between the date of randomization and earliest date of disease progression, and MFS was the duration of survival without invasive loco-regional recurrence, distant metastasis, contralateral breast cancer or second primary breast invasive cancer. For the advanced breast cancer patient population, time to progression and OS were used as primary end points. In the early breast cancer population, MFS and OS were regarded as primary endpoints.

Univariate analysis was conducted on FYN intensity, FYN subcellular localization, tumor size, nodal status, histological type and grade. For multivariate analysis, the Cox proportional hazards regression model was applied to assess the adjusted HR of MFS, OS by subcellular localization of FYN to assess interactions. Factors included in multivariate analysis were tumor size (0–20, 21–30, >30 mm), nodal status (0–4, >4 positive), histological type and grade (ductal grade I, II, III). Survival curves were generated by Kaplan–Meier estimates and P values are calculated by log-rank test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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