**FGFR1 Amplification Drives Endocrine Therapy Resistance and Is a Therapeutic Target in Breast Cancer**

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**Abstract**

Amplification of fibroblast growth factor receptor 1 (FGFR1) occurs in ~10% of breast cancers and is associated with poor prognosis. However, it is uncertain whether overexpression of FGFR1 is causally linked to the poor prognosis of amplified cancers. Here, we show that FGFR1 overexpression is robustly associated with FGFR1 amplification in two independent series of breast cancers. Breast cancer cell lines with FGFR1 overexpression and amplification show enhanced ligand-dependent signaling, with increased activation of the mitogen-activated protein kinase and phosphoinositide 3-kinase–AKT signaling pathways in response to FGF2, but also show basal ligand-independent signaling, and are dependent on FGFR signaling for anchorage-independent growth. FGFR1-amplified cell lines show resistance to 4-hydroxytamoxifen, which is reversed by small interfering RNA silencing of FGFR1, suggesting that FGFR1 overexpression also promotes endocrine therapy resistance. FGFR1 signaling suppresses progesterone receptor (PR) expression in vitro, and likewise, amplified cancers are frequently PR negative, identifying a potential biomarker for FGFR1 activity. Furthermore, we show that amplified cancers have a high proliferative rate assessed by Ki67 staining and that FGFR1 amplification is found in 16% to 27% of luminal B-type breast cancers. Our data suggest that amplification and overexpression of FGFR1 may be a major contributor to poor prognosis in luminal-type breast cancers, driving anchorage-independent proliferation and endocrine therapy resistance.

**Introduction**

Despite substantial improvements in the treatment of breast cancer, resistance to therapy is a major clinical problem. Understanding the mechanisms of resistance and the identification of novel therapeutic targets is of vital importance if the prognosis of breast cancer is to be further improved. Molecular subtyping of breast cancer has identified distinct subtypes of breast cancer. Cancers that express the estrogen receptor (ER) are divided into two broad categories of luminal A and B types (1), largely depending on whether the tumor has low or high proliferation (2, 3). Although, in general, ER-positive tumors are considered to have a good prognosis, highly proliferative, luminal B-type tumors have a poor prognosis in patients treated with adjuvant endocrine therapy (4). Resistance to endocrine therapy, whether acquired or intrinsic, is a major factor implicated in the relapse of these breast cancers, and understanding the factors that result in endocrine therapy resistance is important if outcome is to be improved.

Several recurrent high-level amplifications have been identified in breast cancer (5), and for some of these amplifications, the driver gene has been identified [e.g., HER2 at chromosomal region 17q21 and cyclin D1 (CCND1) at 11q13]. Along with these genes, fibroblast growth factor receptor 1 (FGFR1) was one of the first to be shown to be amplified in breast cancer (6), amplified in ~10% cancers (5). Amplification of FGFR1 is associated with early relapse and poor survival, specifically in ER-positive breast cancer (7). Amplification of FGFR1 is uncommon in HER2-amplified tumors, suggesting that amplification of FGFR1 and HER2 may be mutually exclusive ways of activating similar downstream pathways (7).

However, amplifications at chromosomal region 8p11-12, the genomic locus of FGFR1, are complex with at least two separate regions or cores of amplification (8). Some studies have found that FGFR1 expression correlates with FGFR1 amplification (8–12), but others have not (13–16). Although we have previously shown that the FGFR1-amplified cell line MDA-MB-134 is dependent on FGFR1 for proliferation (17), it is not universally accepted that FGFR1 is a driver of cancers harboring 8p11-12 amplification.
Here, we have comprehensively evaluated $FGFR1$-amplified breast cancer, showing that $FGFR1$ expression is highly correlated with $FGFR1$ copy number. We show that MDA-MB-134 cells have acquired a $KRAS$ mutation that compromises them as a model cell line, and identified several breast cancer cell lines with $FGFR1$ overexpression and amplification. We show that overexpression of $FGFR1$ results in both enhanced ligand-dependent and ligand-independent signaling, with important consequences for anchorage-independent growth and response to endocrine therapy. Finally, we provide evidence that $FGFR1$ amplification is a frequent event in proliferative, luminal B subtype, ER-positive cancers, suggesting that $FGFR1$ overexpression may be a major factor contributing to the poor prognosis of these tumors.

Materials and Methods

Cell lines, materials, and antibodies. Cell lines were obtained from the American Type Culture Collection (ATCC) or Asterand and maintained in phenol red-free DMEM or RPMI 1640 with 10% fetal bovine serum (FBS; FBS Gold, PAA) and 2 mmol/L L-glutamine (Sigma-Aldrich). S68 was a kind gift of Veronique Catros-Quemener (University of Rennes, Rennes, France). MDA-MB-134 was originally obtained directly from M.D. Anderson by Mike O’Hare (Ludwig Institute, London, United Kingdom). PD173074, 4-hydroxytamoxifen (4-OHT), and ICI-182780 were from Sigma, and U0126 was from Calbiochem (Merck KGaA). Small interfering RNA (siRNA) was from Dharmacon: $FGFR1$, siGenome SMARTpool (si$FGFR1$), siGenome Non-Targeting siRNA Pool#1 (si$CON$), and PLK1 siGenome SMARTpool (si$PLK1$). Antibodies used were phosphorylated fibroblast receptor substrate 2 (FRS2; Tyr196), phosphorylated Akt (Ser473), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Thr202/Tyr204), phosphorylated Bsk (Thr339/Ser363), phosphorylated phospholipase C (PLC) γ1 (Tyr783), ERK1/2, CCND1, and progesterone receptor (PR; all from Cell Signaling Technology) and $FGFR1$, ER, and β-actin (all from Santa Cruz Biotechnology).

Tumor samples and microarray-based comparative genomic hybridization. The Guy’s series of 87 ER-positive primary breast cancers all treated with adjuvant tamoxifen ($HER2$) and $KRAS$ mutation that compromises them as a model cell line, and identified several breast cancer cell lines with $FGFR1$ overexpression and amplification. We show that overexpression of $FGFR1$ results in both enhanced ligand-dependent and ligand-independent signaling, with important consequences for anchorage-independent growth and response to endocrine therapy. Finally, we provide evidence that $FGFR1$ amplification is a frequent event in proliferative, luminal B subtype, ER-positive cancers, suggesting that $FGFR1$ overexpression may be a major factor contributing to the poor prognosis of these tumors.

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Tumor samples and microarray-based comparative genomic hybridization. The Guy’s series of 87 ER-positive primary breast cancers all treated with adjuvant tamoxifen has been described previously (3). Analysis of primary breast cancers all treated with adjuvant tamoxifen (aCGH) platform was constructed at the Breakthrough Breast Cancer Research Centre, and arrays were hybridized and analyzed as previously described (19, 20).

Quantitative reverse transcription–PCR. cDNA was synthesized from RNA using SuperScript III and random hexamers (Invitrogen). Quantitative PCR was performed using Taqman chemistry (Applied Biosystems) using standard curve method. Expression of $FGFR1$ (Hs00241111_m1), $PR$ (Hs01556701_m1), or $CCND1$ (Hs00277039_m1) was expressed relative to the mean of three endogenous controls: S18 (4310893E), MRPL19 (Hs00608519_m1), and β-actin (4310881E).

Definition of $FGFR1$ overexpression. Gene expression analysis using Affymetrix U133A Genechips (Affymetrix) was performed and normalized as previously described (3). The median-weighted mean of five $FGFR1$ probes (207822_at, 210973_s_at, 211535_s_at, 222164_at, and 226705_at) was used to assess $FGFR1$ expression.

To define $FGFR1$ overexpression, for both quantitative PCR or Affymetrix data, the SD of the data was estimated from the median absolute deviation. A sample was considered $FGFR1$ overexpressed when level exceeded 3 SDs from the median, identifying samples with outlier overexpression.

CISH and immunohistochemistry. $FGFR1$ CISH was performed on the TMA series with an in-house biotin-labeled probe (21) and on the Guy’s series (3) with $FGFR1$ ZytoDot-SPEC Probe (Zytovision GmbH) and the SPoT-Light CISH Polymer Detection kit (Invitrogen). $FGFR1$ signals per cell were counted in 100 tumor nuclei, considered amplified if >50% of the neoplastic cells harbored either more than five copies of the gene or large gene clusters (22). All amplified tumors were also amplified according to criteria outlined in the American Society of Clinical Oncology/College of American Pathologists guidelines for $HER2$ gene amplification. The Ki67 staining was performed using the MIB-1 clone (Dako). Antigen retrieval was with 2-min pressure cooking in 0.01 mol/L citrate (pH 6). Bound antibody was detected using the Vector ABC kit (Vector Laboratories), with 3,3′-diaminobenzidine as chromogen (Dako). Both $FGFR1$ CISH and Ki67 immunohistochemistry were assessed blinded to $FGFR1$ expression.

Cell line drug sensitivity and siRNA transfection. All experiments were performed in 10% serum unless stated otherwise. Cell lines were transfected with siRNA (50 nmol/L final concentration) in 96-well plates with RNAiMax (Invitrogen). Survival was assessed with CellTiter-Glo cell viability assay (Promega). For sensitivity to PD173074, cell lines were plated in 96-well plates; the following day, media were supplemented with PD173074 at various concentrations; and survival was assessed after 96 h of exposure. For assessment of endocrine therapy sensitivity, cells were maintained in phenol red–free medium supplemented with 10% charcoal/dextran-coated charcoal-stripped serum (HyClone), 2 mmol/L L-glutamine, and 1 nmol/L estradiol (Sigma); plated in 96-well plates; and treated for 6 d with a range of doses of 4-OHT.

$FGFR1$ stable cell lines. cDNA coding for full-length $FGFR1$–IIIc was cloned into the p-LEX-MCS vector (Open Biosystems). The vector was packaged into lentivirus in 293-T cells, and T47D cells were infected with p-LEX-MCS (T47D-EV) or p-LEX-$FGFR1$ (T47D-$FGFR1$). At 96 h after infection, 1 µg/mL puromycin was added, and a polyclonal stable pool was established under continuous selection.

Anchorage-independent growth. CAL120 cells were seeded in 4% agarose (Sigma) in six-well plates (5,000 per well), on a base layer of 5% agarose, in 1× RPMI 1640, 10% FBS, and glutamine with or without 1 µmol/L PD173074. The top layer
was left to set, following which a covering of medium with or without 1 μmol/L PD173074 was added and replaced every 3 to 4 d. After 2 wk, colonies of cells were visualized by light microscopy followed by staining with crystal violet and counting of colony number.

**Western blotting and fluorescence-activated cell sorting.** Indicated cell lines were grown on 10-cm plates, treated as indicated, and lysed in NP40 lysis buffer. Western blots were carried out with precast TA or Bis-Tris gels (Invitrogen) as previously described (23). Fluorescence-activated cell sorting (FACS) analysis was performed as described previously (23).

**ER-directed transcription.** SUM44 cells were transfected with EREIItkLuc and pCH110 using GeneJuice (EMD Biosciences, Inc.), and estrogen response element (ERE)–luciferase and β-galactosidase were assayed after 48 h of FGF2 treatment, or not, as previously described (24).

**Statistical analysis.** All statistical analysis was two-sided and performed with GraphPad Prism version 5.0.

**Results**

**FGFR1 is robustly overexpressed in FGFR1-amplified tumors.** We examined the relationship between FGFR1 amplification and FGFR1 mRNA expression in two independent series of breast cancers. The first series consisted of 87 ER-positive tumors (Guy’s series; ref. 3), with FGFR1 copy number assessed in 58 cases by CISH. FGFR1 mRNA expression, assessed by gene expression profiling, was substantially higher in amplified tumors compared with nonamplified tumors (5.91 versus 1.0; \( P < 0.0001 \), Mann-Whitney U test; Fig. 1A). The second series consisted of invasive breast cancers in a TMA (18). FGFR1 amplification was present in 11.8% tumors

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**Figure 1.** FGFR1-amplified breast tumors and cancer cell lines overexpress FGFR1. A, 58 ER-positive breast cancers distributed in order of FGFR1 mRNA level, expressed relative to the median expression level. Red, tumors with FGFR1 amplification assessed by CISH; black, tumors without FGFR1 amplification. Right, example photomicrographs from a tumor without and with FGFR1 amplification. B, FGFR1 amplification status assessed in a second series of 93 invasive breast cancers. FGFR1 gene expression was assessed by quantitative RT-PCR from FGFR1-amplified tumors, and grade- and ER-matched controls, and expressed relative to the median expression level of controls. FGFR1-amplified tumors had substantially higher median FGFR1 expression than nonamplified controls (13.4 versus 1.0; \( P = 0.0002 \), Mann-Whitney U test). C, FGFR1 expression assessed by quantitative RT-PCR in a panel of 40 breast cancer cell lines. Six cell lines overexpress FGFR1 (indicated by arrows), all of which have high-level FGFR1 amplification (Supplementary Fig. S2). FGFR1 expression displayed relative to median expression. D, Western blot confirming overexpression of FGFR1 protein in cell lines with FGFR1 amplification compared with nonamplified control cell line MCF7.
RNA was extracted from corresponding FFPE tissue sections, and FGFR1 expression was assessed by quantitative PCR in 10 FGFR1-amplified tumor sections and 17 grade- and ER-matched controls. FGFR1 was substantially higher in amplified tumors compared with non-amplified controls (median, 13.4 versus 1.0; \( P < 0.0001 \), Mann-Whitney \( U \) test; Fig. 1B). In both series, overexpression of FGFR1 mRNA was only found in cancers with FGFR1 amplification. In the first (88%, 7 of 8) and second (80%, 8 of 10) series, amplified tumors displayed FGFR1 expression higher than any nonamplified control, validating a tight relationship between amplification and overexpression.

Identification of FGFR1-amplified cell line models. We had previously shown that the MDA-MB-134 cell line, obtained directly from originating lab in M.D. Anderson, harbors FGFR1 amplification and overexpression and was sensitive to FGFR1 inhibitors and silencing of FGFR1 by RNA interference (17). Multiple different siRNAs targeting FGFR1 reduced the survival of MDA-MB-134, showing that the effect was on target (Supplementary Fig. S1). However, over time, we were unable to propagate the original cell line, and we obtained a new sample from the ATCC. To our surprise, the resupplied MDA-MB-134 was no longer sensitive to FGFR1 siRNA (Fig. 2A) despite efficient knockdown of FGFR1 (Supplementary Fig. S1). We confirmed by both aCGH and gene expression profiling that the resupplied line was indeed MDA-MB-134 (Supplementary Fig. S2). The resupplied line overexpressed FGFR1 (Fig. 1D) but was also found to be heterozygous for a G12R KRAS mutation (Supplementary Fig. S1). This mutation has always been present in MDA-MB-134 but originally only at low frequency (25). Blockade of MAP/ERK kinase signaling with U0126 in the KRAS mutant MDA-MB-134 restored dependence on FGFR signaling (Supplementary Fig. S3), suggesting that the KRAS mutation explained the resistance to FGFR targeting.

With MDA-MB-134 partially compromised as a model of FGFR1 amplification, we set out to identify new cell line models of FGFR1 amplification.
models. We screened a panel of 40 breast cancer cell lines by quantitative reverse transcription-PCR (RT-PCR) for \( \text{FGFR1} \) and identified six breast cancer cell lines that overexpressed \( \text{FGFR1} \) (CAL120, JIMT-1, MDA-MB-134 \( \text{KRAS} \), MFM223, S68, and SUM44; Fig. 1C). All cell lines that overexpressed \( \text{FGFR1} \) harbored high-level \( \text{FGFR1} \) amplification as assessed by aCGH (Supplementary Fig. S2). Two cell lines were not investigated further—JIMT-1 (due to coamplification of \( \text{HER2} \)) and MFM223 (due to coamplification of \( \text{FGFR2} \)) (26)—either of which might complicate assessment. All amplified cell lines overexpressed \( \text{FGFR1} \) protein by Western blot (Fig. 1D).

\( \text{FGFR1 amplification is required for anchorage-independent growth.} \) We examined the effect of targeting \( \text{FGFR1} \) in the newly identified cell line models of \( \text{FGFR1} \) amplification. In routine two-dimensional culture, silencing of \( \text{FGFR1} \) by siRNA did not affect survival of SUM44, CAL120, or S68 (Fig. 2B) despite achieving substantial knockdown of \( \text{FGFR1} \) (Fig. 2B). The cell lines were also not sensitive to the potent pan-FGFR inhibitor PD173074 in two-dimensional culture, unlike the \( \text{FGFR2} \)-amplified positive control cell line SUM52PE (Fig. 2C; ref. 27). Therefore, we investigated the requirement of \( \text{FGFR} \) signaling in three-dimensional culture by growing cells in soft agar. This showed that \( \text{FGFR} \) activity was required for anchorage-independent growth of CAL120, and colony formation was completely abolished by PD173074 (Fig. 2D). We were unable to assess S68 and SUM44 in this assay, as they did not grow in soft agar (data not shown).

\( \text{FGFR1 amplification drives both ligand-dependent and ligand-independent signaling.} \) FGFs are present in very low concentrations in normal serum; FGFs bind heparin proteoglycans (HSPG) avidly and are sequestered in the extracellular matrix and cell surface at the site of production (28). We examined downstream signaling in response to the addition of relatively low-dose FGF2 (1 ng/mL) to routine culture medium (Fig. 3A). Signaling through the \( \text{FGFR} \) family is reliant on the adapter protein FRS2 to activate mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)–AKT signaling. Phosphorylation of FRS2 in response to FGF2 was only seen in amplified cell lines (Fig. 3A). Amplified cell lines displayed substantially enhanced ERK1/2 phosphorylation in comparison with control cell lines, with a minor increase in ERK1/2 phosphorylation in MCF7 and no change in SKBR3. Ribosomal S6 kinase (p90-RSK), a downstream effector of MAPK signaling (29–31), was also phosphorylated in response to FGF2 only in amplified cell lines (Supplementary Fig. S4). Phosphorylation of ERK1/2 and AKT was almost exclusively FGF2 dependent in SUM44 (Fig. 3A).

To confirm that overexpression of \( \text{FGFR1} \) can enhance downstream signaling in response to ligand, we generated T47D cells that stably overexpressed \( \text{FGFR1} \). T47D cells were infected with a lentiviral \( \text{FGFR1} \) expression vector, or control empty vector, and a stable polyclonal pool was established. T47D-\( \text{FGFR1} \) cells showed both increased FGF2-dependent signaling, with an induction of AKT phosphorylation not seen in control cells, and an increase in basal-unstimulated signaling (Fig. 3B). Basal-unstimulated signaling in the \( \text{FGFR1} \)-expressing cells was also increased in response to \( \text{FGFR1} \) ligand (Fig. 3B). Basal-unstimulated signaling in the \( \text{FGFR1} \)-expressing cells was also increased in response to \( \text{FGFR1} \) ligand (Fig. 3B). Basal-unstimulated signaling in the \( \text{FGFR1} \)-expressing cells was also increased in response to \( \text{FGFR1} \) ligand (Fig. 3B). Basal-unstimulated signaling in the \( \text{FGFR1} \)-expressing cells was also increased in response to \( \text{FGFR1} \) ligand (Fig. 3B). Basal-unstimulated signaling in the \( \text{FGFR1} \)-expressing cells was also increased in response to \( \text{FGFR1} \) ligand (Fig. 3B). We examined whether \( \text{FGFR1} \) overexpression in a breast cancer cell line showed basal signaling in serum-starved conditions. After serum starvation, phosphorylation of ERK1/2 was blocked by PD173074 in both CAL120 and SUM44PE (Fig. 3C). To differentiate ligand-independent from autocrine-mediated

Figure 3. \( \text{FGFR1} \) amplification drives both ligand-dependent and ligand-independent signaling. A, indicated cell lines growing in 10% serum were treated for 15 min before lysis with 1 ng/mL FGF2 (+) or not (−). Lysates were subjected to SDS-PAGE and Western blotting with antibodies against phosphorylated FRS2 (Tyr196), phosphorylated AKT1 (Ser473), phosphorylated ERK1/2 (Thr202/Tyr204), and β-actin. Two different exposures of FRS2 (Tyr196) are shown. B, stable polyclonal pool of T47D cells was established with empty vector (T47D-EV) or \( \text{FGFR1} \) expression vector (T47D-\( \text{FGFR1} \)). Western blots of T47D-EV or T47D-\( \text{FGFR1} \) cells treated for 15 min before lysis with 1 ng/mL FGF2, or no treatment (−), and blotted with indicated antibodies. C, indicated cell lines were serum starved for 24 h, and lysates were made after 1-h exposure to 1 \( \mu \text{mol/L} \) PD173074 (+), or no exposure (−), as indicated. Lysates were subjected to Western blotting and blotted with indicated antibodies.
signaling, we examined FGF ligand expression in cell lines. CAL120 expressed both FGF2 mRNA and conditioned medium with FGF2 ligand (Supplementary Fig. S4). In contrast, T47D, SUM44, MDA-MB-134, and other FGER1-amplified cell lines did not appreciably express any of the FGER1 ligands (data not shown), indicating that in these cell lines FGER1 overexpression resulted in low-level basal ligand-independent signaling.

**FGER1 amplification drives endocrine therapy resistance.** We have previously shown that amplification of FGER1 is associated with poor prognosis specifically in patients with ER-positive breast cancer (7). One potential explanation for this observation could be resistance to endocrine therapies. To investigate this, we examined the effect of silencing of FGER1 on endocrine therapy sensitivity in the FGER1-amplified ER-positive cell lines. We initially studied the FGER-sensitive MDA-MB-134 subline, which was partially resistant to 4-OHT (Fig. 4A). Silencing of FGER1 in this cell line with FGER1 siRNA SMARTpool, or two individual siRNAs, increased 4-OHT sensitivity compared with cells transfected with siCON nontargeting control (Fig. 4A). At both $10^{-7}$ mol/L and $10^{-6}$ mol/L 4-OHT, siCON-transfected cells were less sensitive than siFGER1-transfected and both individual siRNA-transfected cells ($P < 0.05$, Student’s t test).

SUM44 is an ER-positive cell line that is sensitive to 4-OHT, although addition of FGF2 to medium abolished sensitivity to 4-OHT (Fig. 4B). Silencing of FGER1 in SUM44 only modestly increased sensitivity to 4-OHT but blocked the ability of FGF2 to cause resistance (Fig. 4B), suggesting that the effect of FGF2 was mediated by FGER1. This observation concurs with the strong ligand dependence of downstream signaling in SUM44 (Fig. 3A). FGF2 also induced resistance to the ER antagonist ICI-182780 (data not shown). Similarly, T47D-FGER1 cells showed greater resistance to 4-OHT and estrogen deprivation in response to FGF2, in comparison with T47D-EV cells (Supplementary Fig. S5).

We examined the mechanism of resistance. In SUM44, 4-OHT reduced S-phase fraction, but dual treatment with FGF2 and 4-OHT increased S-phase fraction to levels comparable with untreated cells (Fig. 4C and D). In contrast, in FGER1 siRNA-transfected cells, 4-OHT reduced S-phase fraction, but FGF2 was unable to restore levels comparable with untreated cells [relative increase FGF2 + tamoxifen versus tamoxifen: siCON (120%) versus siFGER1 (43%); $P = 0.047$, Student’s t test]. We examined signaling in SUM44 cells treated with 4-OHT and ICI-182780 with or without FGF2 (Fig. 5). Treatment with FGF2 alone decreased PR expression to a comparable level with 4-OHT. In contrast, CCND1 was substantially elevated in the presence of FGF2 (Fig. 5A) and remained elevated after treatment with 4-OHT. Treatment with ICI-182780 led to ER degradation and partial loss of CCND1 expression in FGF2-treated cells, although CCND1 remained elevated compared with ICI-182780–treated cells without FGF2 (Fig. 5A and B).

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**Figure 4.** FGER1 drives endocrine therapy resistance in amplified lines. A, FGER-sensitive MDA-MB-134 cells, obtained from M.D. Anderson, were transfected with siCON, siFGER1, or two individual siRNA targeting FGER1 (siFGER1-A and siFGER1-B). Starting at 48 h after transfection, cells were treated with range of concentrations of 4-OHT, and survival was assessed after 6 d exposure. Points, mean of three repeat experiments; bars, SE. B, SUM44 cells were transfected with siCON or siFGER1 and, 48 h after transfection, treated with range of concentrations of 4-OHT in the presence of 10 ng/mL FGF2 or no FGF2. Survival was assessed after 6-d exposure. C, propidium iodide FACS profiles in SUM44 cells transfected 6 d earlier with siCON, or siFGER1, and treated for 72 h with 10 ng/mL FGF2, 10 nmol/L 4-OHT, the combination, or no treatment (−). D, quantification of S-phase fraction from three independent experiments. Fraction in S phase: siCON transfected, no treatment (14.4%) versus FGF2/4-OHT treated (13.4%; $P = 0.3$, Student’s t test); siFGER1 transfected, 7.2% versus 4.5% ($P = 0.02$).
Quantitative PCR assessment of PGR mRNA (PR) confirmed that PR expression was suppressed by FGF2 (Fig. 5C). We therefore examined the effect of FGF2 on an ERE-luciferase reporter construct. FGF2 inhibited ER-directed transcription (Fig. 5D), confirming that the decrease in PR expression reflected a suppression of ER-dependent transcriptional activity. Both ERK1/2 and RSK phosphorylation was persistently phosphorylated by FGF2 stimulation after 24 hours, whereas AKT and PLC phosphorylation was undetectable (Fig. 5B). These data suggest that FGFR1 signaling induced endocrine resistance through persistent MAPK activation, which promoted CCND1 expression both in an ER-dependent and ER-independent manner, to cause resistance to endocrine therapy.

Clinical features of FGFR1-amplified tumors. In the Guy’s series of 87 ER-positive tumors all treated with tamoxifen as sole adjuvant therapy (3), distant metastasis-free survival was significantly worse for FGFR1-overexpressing tumors compared with tumors lacking overexpression (Fig. 6A). In this series, FGFR1-overexpressing tumors were frequently ER positive but PR negative (PR negative, 20% nonamplified versus 60% amplified; \( P = 0.032 \), Fisher’s exact test; Fig. 6B). This concurs with the in vitro observations with SUM44 (Fig. 5).

We examined whether FGFR1 amplification/overexpression was associated with any one specific breast cancer subtype (1). We next interrogated a published gene expression array data set of 295 breast cancers from van de Vijver and colleagues (32). Overexpression of FGFR1 was strongly associated with luminal B–type breast cancers (Fig. 6), as was the incidence of 8p11-12 amplification (the genomic locus of FGFR1; Fig. 6). Similarly, in the data set of Chin and colleagues (12), FGFR1 overexpression and high-level amplification of FGFR1 were found specifically in ER-positive tumors (Supplementary Fig. S6). To confirm these findings, we assessed Ki67 expression, as a surrogate of proliferative rate, in the Guy’s series of ER-positive breast cancers. FGFR1-overexpressing cancers had a significantly higher proliferative rate (Fig. 6), with 87.5% (eight of nine) tumors having high Ki67 \( \geq 14\% \) as defined by Cheang and colleagues (2). These data suggest that FGFR1 amplification is particularly important in the highly proliferative, poor-prognosis, luminal B subtype, ER-positive breast cancers.

Figure 5. Signaling in SUM44 cells in response to endocrine therapies. A, Western blots of PR, ER, phosphorylated ERK1/2, ERK1/2, CCND1, \( \beta \)-actin, and FGFR1. SUM44 cell lysates treated for 24 h before lysis with 100 nmol/L 4-OHT, 100 nmol/L ICI-182780, or no treatment (−), with or without 10 ng/mL FGF2. Phosphorylated AKT1 was not detected. B, Western blots of phosphorylated PLCγ1 (Tyr783), phosphorylated AKT, phosphorylated p90-RSK (Thr359/Ser363), phosphorylated ERK1/2, and \( \beta \)-actin on SUM44 cell lysates treated for either 10 min or 24 h with 10 ng/mL FGF2 before lysis. C, quantitative RT-PCR analysis of CCND1 (top) and PR (bottom) expression in SUM44 cells treated with or without 10 ng/mL FGF2 for 24 h before RNA isolation, without (black columns) or in the presence of 100 nmol/L ICI-182780 (gray columns). D, SUM44 cells were cotransfected with EREIItkLuc (ERE-luciferase reporter construct) and pCH110 (\( \beta \)-galactosidase reporter construct) and treated for 48 h with 10 ng/mL FGF2, or no treatment, with 100 nmol/L ICI-182780 as positive control. Luciferase activity was expressed relative to \( \beta \)-galactosidase activity. Columns, mean of three repeats; bars, SE. \( P \) values, Student’s t test.
Discussion

In this study, we have shown that amplification of FGFR1 promotes anchorage-independent proliferation and resistance to endocrine therapies, and this may be reflected in the poor prognosis of amplified cancers. Overexpression of FGFR1 in amplified cell lines results in aberrant ligand-dependent signaling, with persistent activation of MAPK signaling and engagement of PI3K-AKT signaling in response to ligand, which is not seen in unamplified cell lines. In addition, we show that higher levels of FGFR1 expression result in basal ligand-independent signaling, further enhancing downstream signaling. The amplified cell lines MDA-MB-134 and CAL120 are sensitive to targeting of FGFR1, showing that FGFR1 is a potential therapeutic target in amplified cancers.

Our study adds to the increasing evidence linking aberrant FGF signaling to breast cancer (33). A single nucleotide polymorphism in FGFR2 is associated with an increased risk of ER-positive breast cancer (34, 35). Mouse models have shown that expression and constitutive activation of FGFR1 in the mouse mammary epithelium induced proliferation and invasive lesions (36). Activation of the same construct in vitro in murine HC11 cells drove proliferation, survival, and invasion (37), confirming the potential oncogenic nature of FGFR1 signaling. Interestingly, expression of activated FGFR1 in murine prostate induced carcinoma, but a similar FGFR2 construct did not (38), suggesting that FGFR1 may have enhanced oncogenic potential in comparison with FGFR2.

Our data suggest that FGF ligand, potentially in an epithelial-stromal interaction, is important in the promotion of breast cancer progression by FGFR1. FGF2 is expressed at substantially higher levels in breast cancers (39), and in serum and nipple aspirate fluid (40) of patients with breast cancer, compared with women without cancer (40, 41). In addition, breast cancer cells express HSPGs on their cell surface that bind and promote the FGFR1-FGF2 interaction (42). A consequence of
the low FGF ligand concentration of serum is that FGFR1-amplified cancer cell lines have effectively been derived in conditions of very low ligand (43). It is plausible that the lack of dependence on FGFR1 most amplified cell lines display in two-dimensional culture could simply reflect the conditions under which these cell lines were derived.

It is important to emphasize that genes other than FGFR1 in the 8p11-12 amplicon are also likely to contribute to oncogenesis (44–46), potentially acting in collaboration with FGFR1 (46). In addition, FGFR1 is commonly coamplified with CCND1 (5), which may cooperate in oncogenesis (46, 47). We have shown a tight relationship between FGFR1 mRNA expression and FGFR1 amplification. In particular, tumors lacking FGFR1 amplification did not overexpress FGFR1 to a level comparable with amplified tumors, in contrast to previous data (9). A small fraction of FGFR1-amplified cancers do not seem to overexpress FGFR1 mRNA. Although this may constitute a false-positive CISH result, or a false-negative quantitative PCR result, we consider it more likely that these cancers are driven by other genes within the 8p11-12 amplicon (8).

Our data suggest that FGFR1 amplification drives resistance to endocrine therapy in vitro and that this observation is reflected in the poor prognosis of FGFR1-overexpressing tumors treated with adjuvant tamoxifen (Fig. 5). We have also previously studied FGFR1 amplification in a large series of 800 breast cancers (7). In this data set, the ER-positive tumors treated with tamoxifen as sole adjuvant therapy had an equally poor prognosis [FGFR1-amplified overall survival hazard ratio 5.58 versus nonamplified; 95% confidence interval (95% C1), 1.47–21.1]. Although the decision to give tamoxifen was unrandomized in both the data sets (Fig. 6A), the data do not support there being substantial benefit with adjuvant tamoxifen. The observation that ER-positive FGFR1-amplified tumors are more likely to be PR negative than controls (Fig. 6) provides further support for the role of FGFR1 in the growth of these tumors; loss of PR expression is thought to reflect activation of growth factor signaling (48) and may provide a biomarker of FGFR1 activity in amplified cancers.

Taken together, we provide strong circumstantial evidence that FGFR1 amplification is one of the major drivers of highly proliferative, poor-prognosis, luminal B subtype, ER-positive breast cancers. This provides a strong rationale for the investigation of drugs that target FGFR1 in breast cancer, particularly in combination with endocrine therapy. Several studies of FGFR tyrosine kinase inhibitors have commenced, or are planned, in breast cancer and the results of these studies are awaited with interest.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Lesley Ann-Martin for assistance with ERE-luciferase reporter assays and Jonathon Welti and Andrew Reynolds for assistance with FGF2 ELISA.

Grant Support

Cancer Research UK and Breakthrough Breast Cancer. N. Turner is a Cancer Research UK clinician scientist. We acknowledge National Health Service funding to the National Institute for Health Research Biomedical Research Centre.

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Received 10/09/2009; revised 12/03/2009; accepted 12/10/2009; published OnlineFirst 02/23/2010.

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*Cancer Res* 2010;70:2085-2094. Published OnlineFirst February 23, 2010.

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