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Cancer Research

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. *Cancer Res; 70(5); 2085–94. ©2010 AACR.*

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.

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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 (siFGFR1), siGenome Non-Targeting siRNA Pool#1 (siCON), and PLK1 siGenome SMARTpool (siPLK1). Antibodies used were phosphorylated fibroblast receptor substrate 2 (FRS2; Tyr^{196}), phosphorylated AKT1 (Ser^{473}), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Thr^{202}/Tyr^{204}), phosphorylated RSK (Thr^{359}/Ser^{363}), phosphorylated phospholipase C (PLC) γ1 (Tyr^{783}), 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 has been described previously (3). Analysis of *FGFR1* chromogenic *in situ* hybridization (CISH) and Ki67 was on 3-μm-thick formalin-fixed, paraffin-embedded (FFPE) tissue sections. The tissue microarray (TMA) series of 245 invasive breast cancers has been described previously (18). RNA was extracted from tumor sections with RNeasy FFPE RNA Isolation kit (Qiagen). RNA was extracted from cell lines using Trizol (Invitrogen). DNA was extracted using DNeasy blood and tissue kit (Invitrogen). The 32K bacterial artificial chromosome rearray collection (CHORI) tiling path array comparative genomic hybridization (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) on the ABI Prism 7900T System (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

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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

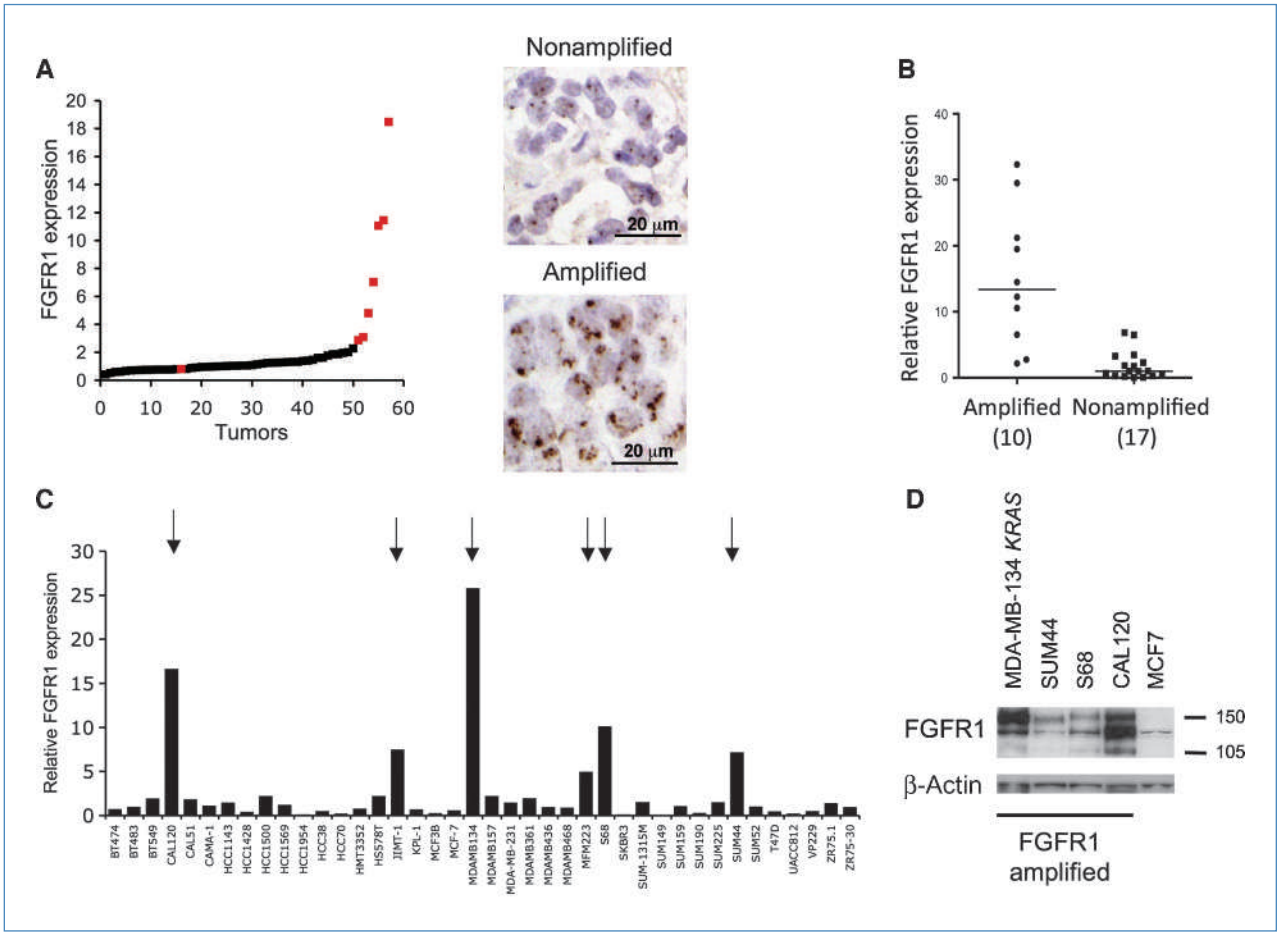

Figure 1. *FGFR1*-amplified breast tumors and cancer cell lines overexpress *FGFR1*. A, 58 ER-positive breast cancers distributed in order of *FGFR1* mRNA level, expressed relative to the median expression level. Red, tumors with *FGFR1* amplification assessed by CISH; black, tumors without *FGFR1* amplification. 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; $P = 0.0002$, 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 off 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.

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(11 of 93 analyzable cores). 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 nonamplified 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

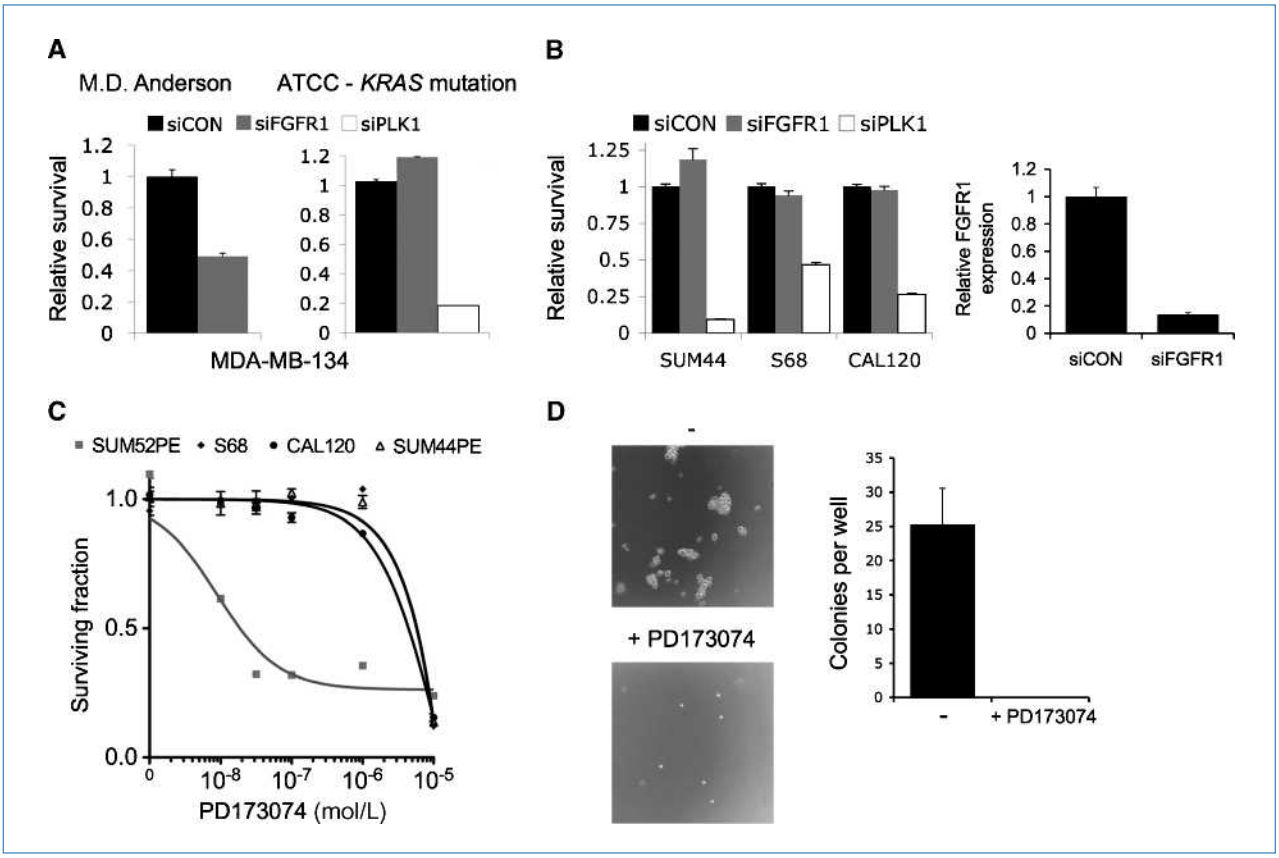

Figure 2. Assessment of the FGFR dependence of *FGFR1*-amplified cell lines. A, sensitivity of MDA-MB-134 cell lines to FGFR1 siRNA (siFGFR1). Cells were transfected with siFGFR1, or siCON nontargeting control, and survival was assessed 6 d later with CellTiter-Glo cell viability assay. MDA-MB-134 cells obtained directly from M.D. Anderson were sensitive to FGFR1 knockdown ($P < 0.001$, Student's *t* test), but not MDA-MB-134 obtained from ATCC. Columns, mean of three repeat experiments; bars, SE. B, left, transfection of *FGFR1*-amplified cell lines with siCON or siFGFR1, and siPLK1 as a positive toxicity control, with survival assessed at 5 to 7 d after transfection; right, FGFR1 expression by quantitative RT-PCR in SUM44 cells transfected with siFGFR1, or siCON, 72 h before RNA extraction. C, *FGFR1*-amplified cell lines were grown for 96 h in medium supplemented with a range of concentrations of PD173074 pan-FGFR tyrosine kinase inhibitor, and survival was expressed relative to that of untreated cells. The SUM52PE breast cancer cell line that harbors *FGFR2* amplification, and is highly sensitive to FGFR inhibitors, was used as a positive control (27). D, CAL120 cells were grown in soft agar with or without continuous exposure to 1 μmol/L PD173074. Example micrographs at 4× power from wells with and without PD173074. Bar chart, mean colonies per well from three repeats [without PD173074 (25.3 colonies) versus with PD173074 (0 colonies); $P = 0.008$, Student's *t* test].

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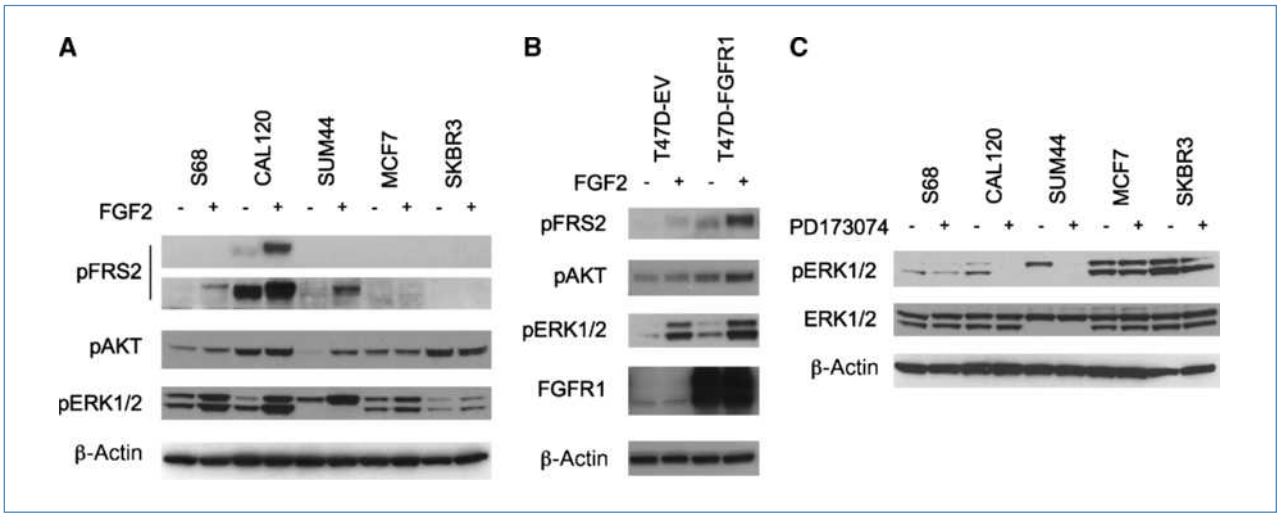

Figure 3. *FGFR1* amplification drives both ligand-dependent and ligand-independent signaling. A, indicated cell lines growing in 10% serum were treated for 15 min before lysis with 1 ng/mL FGF2 (+) or not (–). Lysates were subjected to SDS-PAGE and Western blotting with antibodies against phosphorylated FRS2 (Tyr^{196}), phosphorylated AKT1 (Ser^{473}), phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), and β-actin. Two different exposures of FRS2 (Tyr^{196}) are shown. B, stable polyclonal pool of T47D cells was established with empty vector (T47D-EV) or FGFR1 expression vector (T47D-FGFR1). Western blots of T47D-EV or T47D-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 μmol/L PD173074 (+), or no exposure (–), as indicated. Lysates were subjected to Western blotting and blotted with indicated antibodies.

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

FGFR1 amplification is required for anchorage-independent growth. We examined the effect of targeting FGFR1 in the newly identified cell line models of *FGFR1* amplification. In routine two-dimensional culture, silencing of FGFR1 by siRNA did not affect survival of SUM44, CAL120, or S68 (Fig. 2B) despite achieving substantial knockdown of *FGFR1* (Fig. 2B). The cell lines were also not sensitive to the potent pan-FGFR inhibitor PD173074 in two-dimensional culture, unlike the *FGFR2*-amplified positive control cell line SUM52PE (Fig. 2C; ref. 27). Therefore, we investigated the requirement of FGFR signaling in three-dimensional culture by growing cells in soft agar. This showed that 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).

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 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 FGFR1 can enhance downstream signaling in response to ligand, we generated T47D cells that stably overexpressed FGFR1. T47D cells were infected with a lentiviral FGFR1 expression vector, or control empty vector, and a stable polyclonal pool was established. T47D-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), suggesting that overexpression of FGFR1, to high levels, could induce ligand-independent signaling.

We examined whether *FGFR1* cancer cell lines 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

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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 *FGFR1*-amplified cell lines did not appreciably express any of the FGFR1 ligands (data not shown), indicating that in these cell lines FGFR1 overexpression resulted in low-level basal ligand-independent signaling.

FGFR1 amplification drives endocrine therapy resistance. We have previously shown that amplification of *FGFR1* 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 FGFR1 on endocrine therapy sensitivity in the *FGFR1*-amplified ER-positive cell lines. We initially studied the FGFR-sensitive MDA-MB-134 subline, which was partially resistant to 4-OHT (Fig. 4A). Silencing of FGFR1 in this cell line with FGFR1 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 siFGFR1-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 FGFR1 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 FGFR1. 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-FGFR1 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 FGFR1 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 siFGFR1 (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).

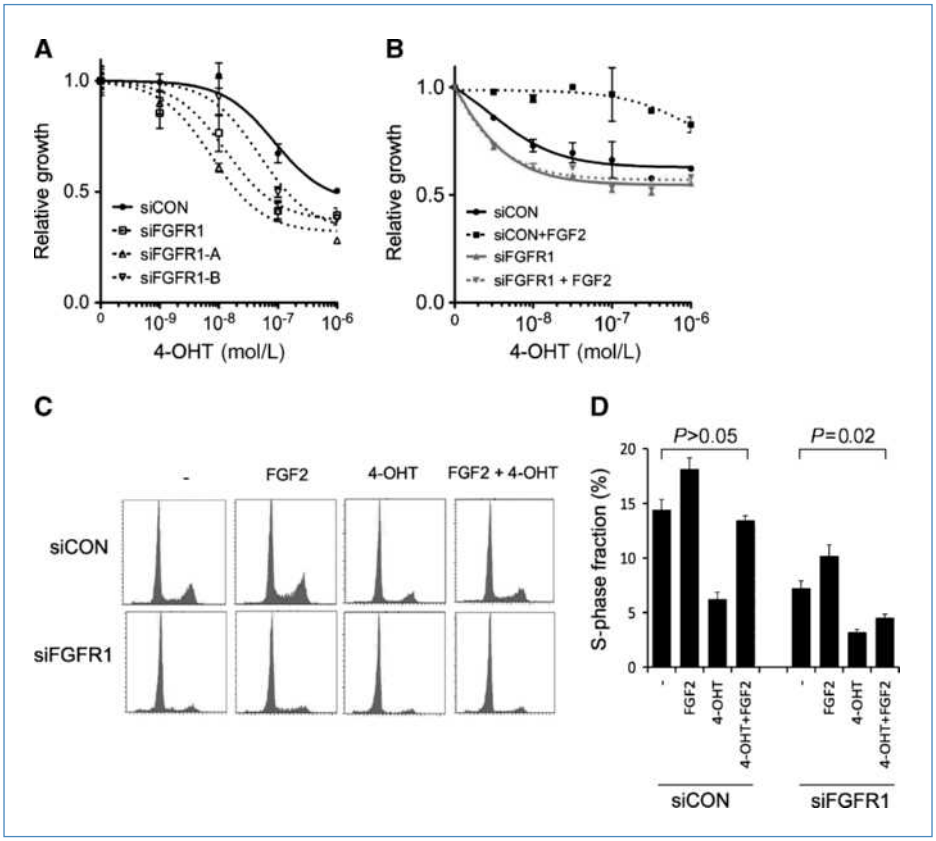

Figure 4. FGFR1 drives endocrine therapy resistance in amplified lines. A, FGFR-sensitive MDA-MB-134 cells, obtained from M.D. Anderson, were transfected with siCON, siFGFR1, or two individual siRNA targeting FGFR1 (siFGFR1-A and siFGFR1-B). Starting at 48 h after transfection, cells were treated with 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 siFGFR1 and, 48 h after transfection, treated with range of concentrations of 4-OHT in the presence of 10 ng/mL FGF2 or with no FGF2. Survival was assessed after 6-d exposure. C, propidium iodide FACS profiles in SUM44 cells transfected 6 d earlier with siCON, or siFGFR1, 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); siFGFR1 transfected, 7.2% versus 4.5% ($P = 0.02$).

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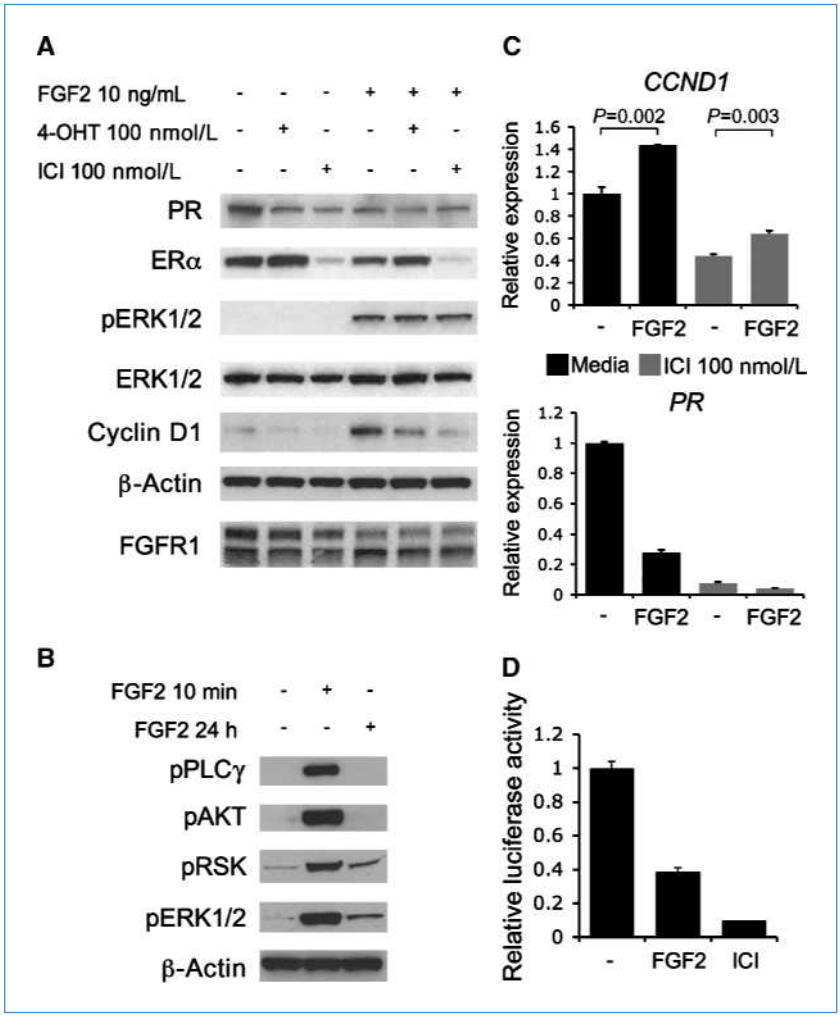

Figure 5. Signaling in SUM44 cells in response to endocrine therapies. A, Western blots of PR, ER, phosphorylated ERK1/2, ERK1/2, CCND1, β-actin, and FGFR1. SUM44 cell lysates treated for 24 h before lysis with 100 nmol/L 4-OHT, 100 nmol/L ICI-182780, or no treatment (–), with or without 10 ng/mL FGF2. Phosphorylated AKT1 was not detected. B, Western blots of phosphorylated PLCγ1 (Tyr^{783}), phosphorylated AKT, phosphorylated p90-RSK (Thr^{359}/Ser^{363}), phosphorylated ERK1/2, and β-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 (β-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 β-galactosidase activity. Columns, mean of three repeats; bars, SE. *P* values, Student's *t* test.

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 [≥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.

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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

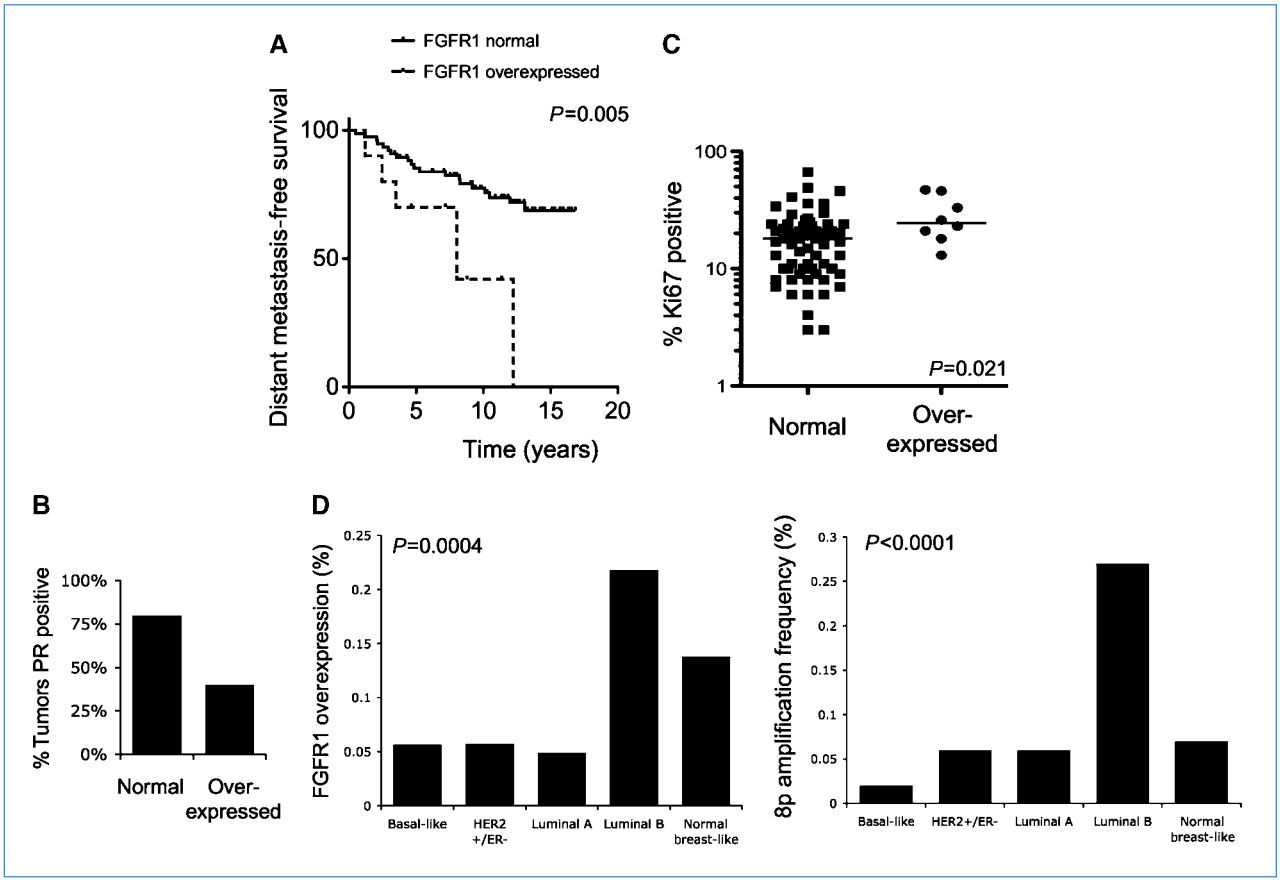

Figure 6. *FGFR1* overexpression is common in high-risk ER-positive breast cancer. A, Kaplan-Meier curves of distant metastasis-free survival from Guy's series of ER-positive tumors with *FGFR1* overexpression (n = 10) versus normal *FGFR1* expression (76). *FGFR1*-overexpressing tumors have substantially worse survival (hazard ratio, 7.4; 95% CI, 1.8–30.5; P = 0.0053, log-rank test). B, proportion of tumors with PR expression in the same cohort (all tumors were ER positive; P = 0.03, Fisher's exact test). C, Ki67 was assessed by immunohistochemistry in the same cohort. *FGFR1*-overexpressing tumors have higher Ki67 (P = 0.021, Mann-Whitney U test). Of tumors with high proliferation [≥14% Ki67 as a surrogate for luminal B subtype (2)], 16% have *FGFR1* overexpression compared with 3.5% of low-proliferating cancers. D, left, incidence of *FGFR1* overexpression in breast cancers according to intrinsic subtype (23% luminal B overexpress *FGFR1*); right, incidence of 8p11-12 amplification, defined by co-overexpression of neighboring genes (Supplementary Materials and Methods), according to intrinsic subtype (27% luminal B have 8p11-12 amplification). Analysis of data on 295 invasive breast cancers from van de Vijver and colleagues (32). Statistical comparison across groups was with the χ^2 test.

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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% CI), 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.

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

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