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## ***FGFR1* amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer**

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### **Abstract**

Amplification of *FGFR1* occurs in approximately 10% of breast cancers and is associated with poor prognosis. However, it is uncertain whether over-expression of FGFR1 is causally linked to the poor prognosis of amplified cancers. Here, we show that *FGFR1* over-expression is robustly associated with *FGFR1* amplification in two independent series of breast cancers. Breast cancer cell lines with *FGFR1* over-expression, and amplification, show enhanced ligand dependent signalling, with increased activation of the MAPK and PI3K-AKT signalling pathways in response to FGF2, but also show basal ligand independent signalling, and are dependent on FGFR signalling for anchorage independent growth. *FGFR1* amplified cell lines demonstrate resistance to 4-OH-Tamoxifen, that is reversed by siRNA silencing of FGFR1, suggesting that FGFR1 over-expression also promotes endocrine therapy resistance. FGFR1 signalling 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 16-27% of luminal B type breast cancers. Our data suggests that amplification and over-expression of *FGFR1* may be a major contributor to poor prognosis in luminal type breast cancers, driving anchorage independent proliferation and endocrine therapy resistance.

### **Keywords**

Breast Cancer; FGFR1; Endocrine therapy

### **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

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further improved. Molecular subtyping of breast cancer has identified distinct subtypes of breast cancer. Cancers that express the oestrogen receptor (ER) divide into two broad categories of luminal A and B types(1), largely depending on whether the tumour has low or high proliferation respectively(2, 3). Although, in general, ER positive tumours are considered to have a good prognosis, highly proliferative, luminal B type tumours 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.

A number of recurrent high-level amplifications have been identified in breast cancer(5), and for some of these amplifications the driver gene has been identified, for example *HER2* at chromosomal region 17q21 and *CCND1* and 11q13. Along with these genes, *FGFR1* was one of the first to be shown to be amplified in breast cancer(6), amplified in approximately 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 tumours, 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 demonstrated 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 harbouring 8p11-12 amplification.

Here, we have comprehensively evaluated *FGFR1* amplified breast cancer, demonstrating 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 a number of breast cancer cell lines with FGFR1 over-expression and amplification. We show that over-expression of FGFR1 results in both enhanced ligand dependent, and independent, signalling with important consequences for anchorage independent growth and response to endocrine therapy. Finally we provide evidence that *FGFR1* amplification is a frequent in proliferative, luminal B subtype ER positive cancers, suggesting that FGFR1 over-expression may be a major factor contributing to the poor prognosis of these tumours.

## Materials and Methods

### Cell lines, materials and antibodies

Cell lines were obtained from ATCC or Asterand, and maintained in phenol red free DMEM or RPMI with 10% FBS (PAA gold) and 2mM L-glutamine (Sigma-Aldrich, Dorset, UK). S68 was a kind gift of Veronique Catros, Rennes, France. MDA-MB-134 was originally obtained directly from MD Anderson by Mike O' Hare, Ludwig Institute, London. PD173074, 4-OH-Tamoxifen (4-OHT) and ICI-182780 were from Sigma and U0126 Calbiochem (Merck KGaA, Darmstadt, Germany). siRNA were from Dharmacon (Lafayette, CO): FGFR1 siGenome SMARTpool (siFGFR1,M-003131-03), siGenome Non-Targeting siRNA Pool#1 (siCON,D-001206-13), and PLK1 siGenome SMARTpool (siPLK1,M-003290-01). Antibodies used were phosphorylated FRS2-Tyr196 (3864), phosphorylated AKT1-Ser473 (4058), phosphorylated ERK1/2-Thr202/Tyr204 (4370), phosphorylated RSK-Thr359/Ser363 (9344), phosphorylated PLCgamma1-Tyr783 (2821), ERK1/2 (9102), CCND1 (2978), PR (3172) (all Cell Signaling Technology, Danvers, MA),

and FGFR1 (sc-121), ER (sc-543),  $\beta$ -ACTIN (sc-1616) (all Santa-Cruz Biotechnology, Santa Cruz, CA).

### Tumour samples and Microarray-Based Comparative Genomic Hybridisation,

The Guy's series of 87 oestrogen receptor positive primary breast cancers all treated with adjuvant tamoxifen has been described previously(3). Analysis of *FGFR1* CISH and Ki67 was on 3 $\mu$ M 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 tumour sections with RNeasy FFPE RNA Isolation Kit (Qiagen, Crawley, UK). RNA was extracted from cell lines using Trizol (Invitrogen, Paisley, UK). DNA was extracted using DNeasy blood and tissue kit (Invitrogen). The 32K BAC re-array collection (CHORI) tiling path aCGH platform was constructed at the Breakthrough Breast Cancer Research Centre, and arrays hybridised and analysed as previously described(19, 20).

### Quantitative RT-PCR

cDNA was synthesised from RNA using Superscript III and random hexamers (Invitrogen). Quantitative PCR was performed using Taqman chemistry (Applied Biosystems, Foster City, CA) 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  $\beta$ -ACTIN (4310881E).

### Definition of FGFR1 over-expression

Gene expression analysis using Affymetrix U133A Genechips (Affymetrix, Santa Clara, CA) was performed and normalised as previously described(3). The median-weighted mean of five *FGFR1* probes (207822\_at, 210973\_s\_at, 211535\_s\_at, 222164\_at, 226705\_at) was used to assess *FGFR1* expression.

To define *FGFR1* over-expression, for both quantitative PCR or Affymetrix data, the standard deviation of the data was estimated from the median absolute deviation. A sample was considered *FGFR1* over-expressed when level exceeded 3 standard deviations from the median, identifying samples with outlier over-expression.

### Chromogenic in situ hybridisation (CISH) and Immunohistochemistry

*FGFR1* CISH was performed on the TMA series with an in-house biotin-labelled probe(21), and on the Guy's series(3) with *FGFR1* ZytoDot-SPEC Probe (Zytovision GmbH, Bremerhaven, Germany) and the SPoT-Light CISH Polymer Detection Kit (Invitrogen). *FGFR1* signals per cell were counted in 100 tumour nuclei, considered amplified if >50% of the neoplastic cells harboured either >5 copies of the gene or large gene clusters(22). All amplified tumours were also amplified according to criteria outlined in the ASCO/CAP guidelines for *HER2* gene amplification. The Ki67 staining was performed using the MIB-1 clone (Dako, code M7240). Antigen retrieval was with 2 minutes pressure cooking in 0.01M citrate pH6. Bound antibody was detected using the Vector ABC kit (Vector laboratories, PK-7200), with DAB as chromogen (K3468, Dako). Both *FGFR1* CISH and Ki67 IHC 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 (50nM final concentration) in 96 well plates with RNAiMax (Invitrogen). Survival was assessed with Cell Titre-Glo cell viability assay (Promega,

Madison, WI). For sensitivity to PD173074, cell lines were plated in 96 well plates, the following day media supplemented with PD173074 at increasing concentrations, and survival assessed after 96 hours exposure. For assessment of endocrine therapy sensitivity cells were maintained in Phenol Red free media, supplemented with 10% charcoal/DCC stripped serum (HyClone, South Logan, UT), 2mM L-glutamine, and 1nM oestradiol (Sigma), plated in 96 well plates and treated for 6 days 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, Huntsville, AL). 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 hour post infection 1µg/ml puromycin was added, and a polyclonal stable pool established under continuous selection.

### Anchorage independent growth

CAL120 cells were seeded in 4% agarose (Sigma) in 6 well plates (5000cells per well), on a base layer of 5% agarose, in 1X RPMI, 10% FBS, and glutamine with or without 1µM PD173074. The top layer was left to set, following which a covering of media with or without 1µM PD173074 was added, and replaced every 3-4 days. After two weeks growth colonies of cells were visualised by light microscopy, followed by staining with crystal violet and counting of colony number.

### Western blotting and FACS

Indicated cell lines were grown on 10cm 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). FACS analysis was performed as described previously (23).

### ER-directed transcription

SUM44 were transfected with EREII<sub>tk</sub>Luc and pCH110 using GeneJuice (EMD Biosciences, Inc., Madison, WI USA), and ERE-luciferase and β-galactosidase assayed after 48 hrs FGF2 treatment, or not, as previously described(24).

### Statistical analysis

All statistical analysis was two sided and performed with GraphPab Prism version 5.0.

## Results

### FGFR1 is robustly over-expressed in FGFR1 amplified tumours

We examined the relationship between *FGFR1* amplification and *FGFR1* mRNA expression in two independent series of breast cancers. The first series consisted of 87 oestrogen (ER) positive tumours (Guy's series)(3), with *FGFR1* copy number assessed in 58 cases by chromogenic *in situ* hybridisation (CISH). *FGFR1* mRNA expression, assessed by gene expression profiling, was substantially higher in amplified tumours compared to non-amplified tumours (5.91 vs 1.0 respectively,  $p < 0.0001$  Mann Whitney U Test) (Figure 1A). The second series consisted of invasive breast cancers in a tissue microarray (TMA)(18). *FGFR1* amplification was present in 11.8% tumours (11/93 analysable cores). RNA was extracted from corresponding formalin fixed paraffin embedded tissue sections and *FGFR1* expression assessed by quantitative PCR, in 10 *FGFR1* amplified tumour sections and 17 grade and ER matched controls. *FGFR1* was substantially higher in amplified tumours compared to non-amplified controls (Median 13.4 vs 1.0 respectively,  $p < 0.0001$  Mann Whitney U Test) (Figure 1B). In both series over-expression of *FGFR1* mRNA was only

found in cancers with *FGFR1* amplification. In the first series 88% (7/8), and the second series 80% (8/10), amplified tumours displayed *FGFR1* expression higher than any non-amplified control, validating a tight relationship between amplification and over-expression.

### Identification of *FGFR1* amplified cell line models

We had previously shown that the MDA-MB-134 cell line, obtained directly from originating lab in MD Anderson, harbours *FGFR1* amplification and over-expression, and was sensitive to *FGFR1* inhibitors and silencing of *FGFR1* by RNA interference (siRNA) (17). Multiple different siRNA targeting *FGFR1* reduced the survival of MDA-MB-134, demonstrating that the effect was on-target (Supplementary Figure 1). However, over time we were unable to propagate the original cell line, and we obtained a new sample from the American Type Culture Collection (ATCC). To our surprise, the re-supplied MDA-MB-134 was no longer sensitive to *FGFR1* siRNA (Figure 2A), despite efficient knockdown of *FGFR1* (Supplementary Figure 1). We confirmed by both array CGH and gene expression profiling that the re-supplied line was indeed MDA-MB-134 (Supplementary Figure 3). The re-supplied line over-expressed *FGFR1* (Figure 1D), but was also found to be heterozygous for a G12R *KRAS* mutation (Supplementary Figure 1). This mutation has always been present in MDA-MB-134, but originally only at low frequency (25). Blockade of MEK signalling with U0126 in the *KRAS* mutant MDA-MB-134 restored dependence on *FGFR* signalling (Supplementary Figure 2), 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. We screened a panel of 40 breast cancer cell lines by quantitative RT-PCR for *FGFR1*, and identified six breast cancer cell lines that over-expressed *FGFR1* (CAL120, JIMT-1, MDA-MB-134 *KRAS*, MFM223, S68, and SUM44, Figure 1C). All cell lines that over-expressed *FGFR1* harboured high level *FGFR1* amplification as assessed by arrayCGH (Supplementary Figure 3). Two cell lines were not investigated further: JIMT-1 due to co-amplification of *HER2*, and MFM223 due to co-amplification of *FGFR2* (Turner et al submitted), either of which might complicate assessment. All amplified cell lines over-expressed *FGFR1* protein by western blot (Figure 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 2D culture, silencing of *FGFR1* by siRNA did not effect survival of SUM44, CAL120, or S68 (Figure 2B) despite achieving substantial knockdown of *FGFR1* (Figure 2B). The cell lines were also not sensitive to the potent pan-*FGFR* inhibitor PD173074 in 2D culture, unlike the *FGFR2* amplified positive control cell line SUM52PE(26) (Figure 2C). Therefore, we investigated the requirement of *FGFR* signalling in 3D culture by growing cells in soft agar. This demonstrated that *FGFR* activity was required for anchorage independent growth of CAL120, and colony formation was completely abolished by PD173074 (Figure 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 independent signalling

Fibroblast growth factors (FGFs) are present in very low concentrations in normal serum; FGFs bind heparin proteoglycans (HSPGs) avidly and are sequestered in the extracellular matrix and cell surface at the site of production (27). We examined down-stream signalling in response to the addition of relatively low dose FGF2 (1ng/ml) to routine culture media (Figure 3A). Signalling through the *FGFR* family is reliant on the adapter protein Fibroblast Receptor Substrate 2 alpha (FRS2) to activate MAPK and PI3K-AKT signalling.



Phosphorylation of FRS2 in response to FGF2 was only seen in amplified cell lines (Figure 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 (RSK), a down stream effector of MAPK signalling(28-30), was also phosphorylated in response to FGF2 only in amplified cell lines (Supplementary Figure 4). Phosphorylation of ERK1/2 and AKT was almost exclusively FGF2 dependent in SUM44 (Figure 3A).

To confirm that over-expression of FGFR1 can enhance downstream signalling in response to ligand, we generated T47D cells that stably over-expressed FGFR1. T47D were infected with a lentiviral FGFR1 expression vector, or control empty vector, and a stable polyclonal pool established. T47D-FGFR1 cells demonstrated both increased FGF2 dependent signalling, with an induction of AKT phosphorylation not seen in control cells, but also an increase in basal-unstimulated signalling (Figure 3B) suggesting that over-expression of FGFR1, to high levels, could induce ligand independent signalling.

We examined whether *FGFR1* cancer cell lines demonstrated basal signalling in serum starved conditions. After serum starvation, phosphorylation of ERK1/2 was blocked by PD173074 in both CAL120 and SUM44PE (Figure 3C). To differentiate ligand independent from autocrine mediated signalling, we examined for FGF ligand expression in cell lines. CAL120 expressed both FGF2 mRNA and conditioned media with FGF2 ligand (Supplementary Figure 4). 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 over-expression resulted in low-level basal ligand independent signalling.

### 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 sub-line, which was partially resistant to 4-HydroxyTamoxifen (4-OHT) (Figure 4A). Silencing of FGFR1 in this cell line with FGFR1 siRNA SMARTpool, or two individual siRNA, increased 4-OHT sensitivity compared to cells transfected with siCON non-targeting control (Figure 4A). At both  $10^{-7}$ M and  $10^{-6}$ M 4-OHT, siCON transfected cells were less sensitive than siFGFR1, 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 media abolished sensitivity to 4-OHT (Figure 4B). Silencing of FGFR1 in SUM44 only modestly increased sensitivity to 4-OHT but blocked the ability of FGF2 to cause resistance (Figure 4B), suggesting that the effect of FGF2 was mediated by FGFR1. This observation concurs with the strong ligand dependence of downstream signalling in SUM44 (Figure 3A). FGF2 also induced resistance to the oestrogen receptor antagonist ICI-182780 (data not shown). Similarly, T47D-FGFR1 cells showed greater resistance to 4-OHT and oestrogen deprivation in response to FGF2, in comparison with T47D-EV cells (Supplementary Figure 5).

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 (Figure 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 vs tamoxifen: siCON 120% vs siFGFR1 43%

p=0.047, Student's T test). We examined signalling in SUM44 cells treated with 4-OHT and ICI-182780, with or without FGF2 (Figure 5). Treatment with FGF2 alone decreased progesterone receptor (PR) expression, to a comparable level to 4-OHT. In contrast, cyclin D1 (CCND1) was substantially elevated in the presence of FGF2 (Figure 5A), and remained elevated after treatment with 4-OHT. Treatment with ICI-182780 lead to ER degradation and partial loss of CCND1 expression in FGF2 treated cells, although CCND1 remained elevated compared to ICI-182780 treated cells without FGF2 (Figure 5A/B).

Quantitative PCR assessment of *PGR* mRNA (*PR*) confirmed that *PR* expression was suppressed by FGF2 (Figure 5C). We, therefore, examined the effect of FGF2 on an ERE (estrogen response element) luciferase reporter construct. FGF2 inhibited ER-directed transcription (Figure 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 24hr, whereas AKT and phospholipase C phosphorylation was undetectable (Figure 5B). This data suggests that FGFR1 signalling induced endocrine resistance through persistent MAPK activation, which promoted CCND1 expression both in an ER dependent and independent manner to cause resistance to endocrine therapy.

### Clinical features of FGFR1 amplified tumours

In the Guy's series of 87 ER positive tumours all treated with tamoxifen as sole adjuvant therapy(3), distant metastasis free survival was significantly worse for *FGFR1* over-expressing tumours compared to tumours lacking over-expression (Figure 6A). In this series, *FGFR1* over-expressing tumours were frequently ER positive but PR negative (PR negative 20% non-amplified vs 60% amplified, p=0.032 Fisher's exact test) (Figure 6B). This concurs with the *in vitro* observations with SUM44 (Figure 5).

We examined whether *FGFR1* amplification / over-expression 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 et al(31). Over-expression of *FGFR1* was strongly associated with luminal B type breast cancers (Figure 6D), as was the incidence of 8p11-12 amplification (the genomic locus of *FGFR1*, Figure 6D). Similarly, in the data set of Chin et al (12), *FGFR1* over-expression and high level amplification of *FGFR1* was found specifically in ER positive tumours (Supplementary Figure 6). 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* over-expressing cancers had a significantly higher proliferative rate (Figure 6C) with 87.5% (8/9) tumours having high Ki67 ( 14% as defined by Cheang et al 2009(2)). These data suggests that *FGFR1* amplification is particularly important in the high proliferative, poor prognosis, luminal B subtype of ER positive breast cancers.

### 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. Over-expression of FGFR1 in amplified cell lines results in aberrant ligand dependent signalling, with persistent activation of MAPK signalling and engagement of PI3K-AKT signalling in response to ligand, which is not seen in un-amplified cell lines. In addition, we show that higher levels FGFR1 expression result in basal ligand independent signalling further enhancing down-stream signalling. The amplified cell lines MDA-MB-134 and CAL120 are sensitive to targeting of FGFR1, demonstrating that FGFR1 is a potential therapeutic target in amplified cancers.

Our study adds to the increasing evidence linking aberrant FGF signalling to breast cancer(32). A single nucleotide polymorphism in *FGFR2* is associated with an increased risk of ER positive breast cancer(33, 34). Mouse models have demonstrated that expression and constitutive activation of FGFR1 in the mouse mammary epithelium induced proliferation and invasive lesions(35). Activation of the same construct *in vitro* in murine HC11 cells drove proliferation, survival, and invasion(36), confirming the potential oncogenic nature of FGFR1 signalling. Interestingly, expression of activated FGFR1 in murine prostate induced carcinoma, but a similar FGFR2 construct did not(37), 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(38), and in serum and nipple aspirate fluid(39) of patients with breast cancer compared to women without cancer(39, 40). In addition, breast cancer cells express HSPGs on their cell surface that bind and promote the FGFR1-FGF2 interaction(41). A consequence of the low FGF ligand concentration of serum is that *FGFR1* amplified cancer cell lines have effectively been derived in conditions of either very low ligand (42). It is plausible, that the lack of dependence on FGFR1 most amplified cell lines display in 2D culture could simply reflect the conditions under which these cell lines were derived.

It is important to emphasise that genes other than *FGFR1* in the 8p11-12 amplicon are also likely to contribute to oncogenesis(43-45), potentially acting in collaboration with *FGFR1*(45). In addition, *FGFR1* is commonly co-amplified with *CCND1*(5), which may cooperate in oncogenesis(45, 46). We have shown a tight relationship between *FGFR1* mRNA expression and *FGFR1* amplification. In particular, tumours lacking *FGFR1* amplification did not over-express *FGFR1* to a level comparable with amplified tumours, in contrast to previous data(9). A small fraction of *FGFR1* amplified cancers do not appear to over-express *FGFR1* mRNA. Although this may constitute a false positive CISH result, or a false negative qPCR result, we consider it more likely that these cancers are driven by other genes within the 8p11-12 amplicon(8).

Our data suggests that *FGFR1* amplification drives resistance to endocrine therapy *in vitro*, and that this observation is reflected in the poor prognosis of *FGFR1* over-expression tumours treated with adjuvant tamoxifen (Figure 5). We have also previously studied *FGFR1* amplification in a large series of 800 breast cancers(7). In this data set, the ER positive tumours treated tamoxifen as sole adjuvant therapy had an equally poor prognosis (*FGFR1* amplified overall survival HR 5.58, 95% CI 1.47 - 21.1). Although the decision to give tamoxifen was unrandomised in both the data sets (Figure 6A), the data does not support there being substantial benefit with adjuvant tamoxifen. The observation that ER positive *FGFR1* amplified tumours are more likely to be PR negative than controls (Figure 6) provides further support for the role of FGFR1 in the growth of these tumours; loss of PR expression is thought to reflect activation of growth factor signalling(47) 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, in particular in combination with endocrine therapy. A number of studies of FGFR tyrosine kinase inhibitors have commenced, or are planned, in breast cancer and the results of these studies are awaited with interest.



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

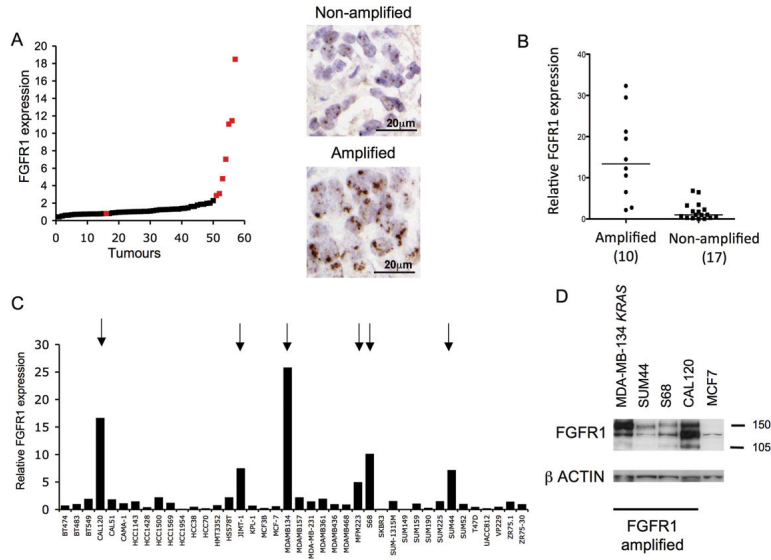
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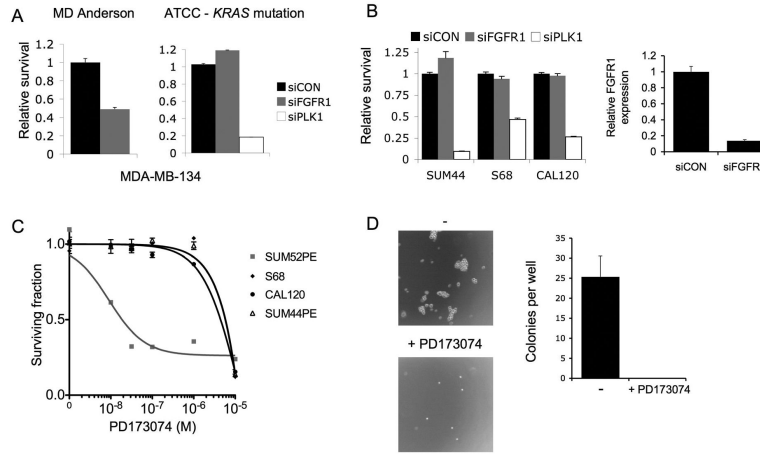
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**Figure 1. *FGFR1* amplified breast tumours and cancer cell lines over-express *FGFR1***  
**A.** 58 ER positive breast cancers distributed in order of *FGFR1* mRNA level, expressed relative to the median expression level; Red - tumours with *FGFR1* amplification assessed by CISH; Black - tumours without *FGFR1* amplification. Right panel example photomicrographs from a tumour 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 tumours, and grade and ER matched controls, and expressed relative to the median expression level of controls. *FGFR1* amplified tumours had substantially higher median *FGFR1* expression than non-amplified controls (13.4 vs 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 over-express *FGFR1* (indicated by arrows), all off which have high level *FGFR1* amplification (Supplementary Figure 3). *FGFR1* expression displayed relative to median expression.  
**D.** Western blot confirming over-expression of FGFR1 protein in cell lines with *FGFR1* amplification compared to non-amplified control cell line MCF7.



**Figure 2. Assessment of the FGFR dependence of *FGFR1* amplified cell lines**

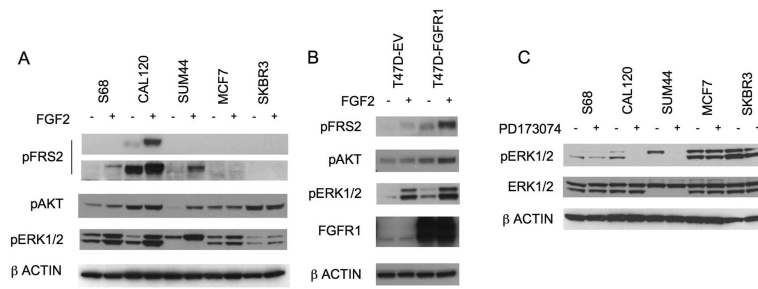
**A.** Sensitivity of MDA-MB-134 cell lines to FGFR1 siRNA (siFGFR1). Cells were transfected with siFGFR1, or siCON non-targeting control, and survival assessed six days later with Cell Titre-Glo<sup>®</sup> cell viability assay (Promega). MDA-MB-134 obtained directly from MD Anderson were sensitive to FGFR1 knockdown ( $p < 0.001$ , Student's T Test), but not MDA-MB-134 obtained from ATCC. Error bars SEM of three repeat experiments.

**B.** Left: Transfection of *FGFR1* amplified cell lines with siCON or siFGFR1, and siPLK1 as a positive toxicity control, with survival assessed at 5-7 days transfection. Right: FGFR1 expression by quantitative RT-PCR in SUM44 cells transfected with siFGFR1, or siCON, 72 hours prior to RNA extraction.

**C.** *FGFR1* amplified cell lines were grown for 96 hrs in media supplemented with a range of concentrations of PD173074 pan FGFR tyrosine kinase inhibitor, and survival expressed relative to that of untreated cells. The SUM52PE breast cancer cell line that harbours *FGFR2* amplification, and is highly sensitive to FGFR inhibitors, was used a positive control (26).

**D.** CAL120 cells were grown in soft agar with or without continuous exposure to 1 $\mu$ M PD173074. Example micrographs at 4X power from wells with and without PD173074. Bar chart: mean colonies per well from 3 repeats (without 25.3 colonies vs with PD173074 0 colonies,  $p = 0.008$ , Student's T test).



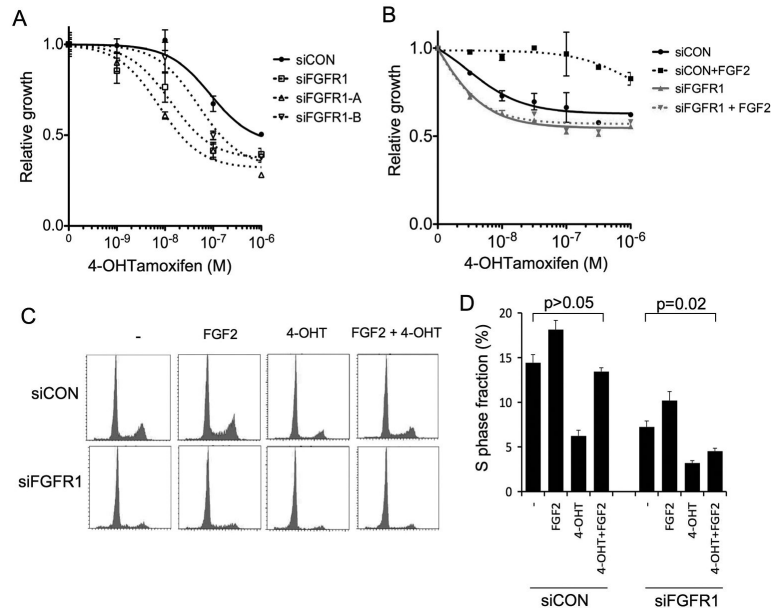


**Figure 3. *FGFR1* amplification drives both ligand dependent and independent signalling**

**A.** Indicated cell lines growing in 10% serum were treated for 15 minutes prior to lysis with 1ng/ml FGF2 (+), or not (-). Lysates were subject to SDS-PAGE and western blotting with antibodies against phosphorylated-FRS2-Tyr196, phosphorylated-AKT1-Ser473, phosphorylated-ERK1/2-Thr202/Tyr204, and  $\beta$ -ACTIN. Two different exposures of FRS2-Tyr196 are shown.

**B.** Stable polyclonal pool of T47D cells were established with empty vector (T47D-EV) or FGFR1 expression vector (T47D-FGFR1). Western blots of T47D-EV or T47D-FGFR1 cells treated for 15 minutes prior to lysis with 1ng/ml FGF2, or no treatment (-), and blotted with indicated antibodies.

**C.** Indicated cell lines were serum starved for 24 hours, and lysates were made after 1hr exposure to 1 $\mu$ M PD173074 (+), or no exposure (-), as indicated. Lysates were subjected to western blotting and blotted with indicated antibodies.



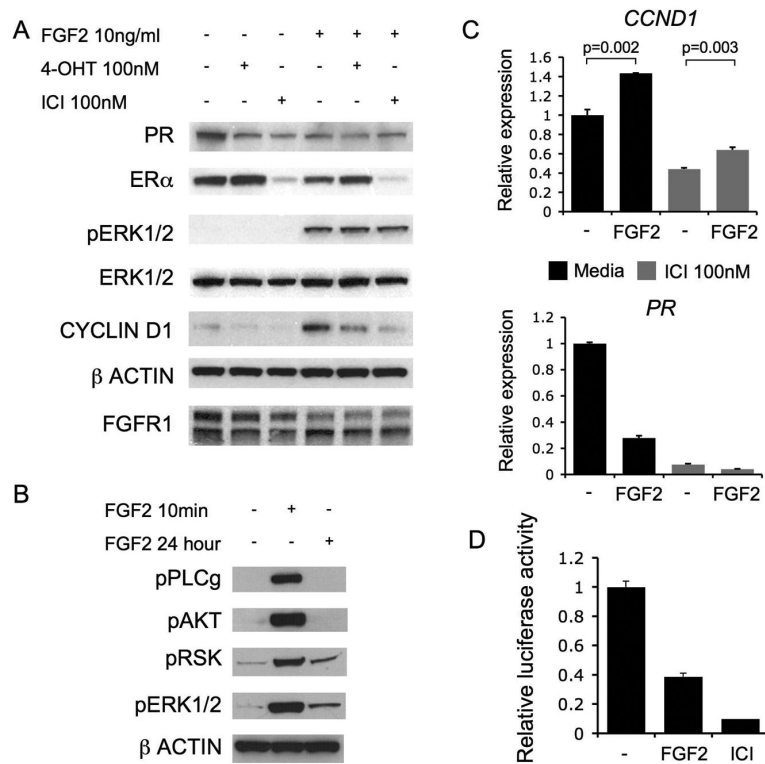
**Figure 4. FGFR1 drives endocrine therapy resistance in amplified lines**

**A.** FGFR sensitive MDA-MB-134 cells, obtained from MD Anderson, were transfected with siCON, siFGFR1, or two individual siRNA targeting FGFR1 (siFGFR1-A and siFGFR1-B). Starting at 48hours post transfection, cells were treated with range of concentrations of 4-hydroxytamoxifen (4-OHT), and survival assessed at 6 days exposure. Error bars represent SEM of 3 repeat experiments.

**B.** SUM44 cells were transfected with siCON, or siFGFR1, and 48 hours after transfection treated with range of concentrations of 4-OHT in the presence of 10ng/ml FGF2, or with no FGF2. Survival was assessed after 6 days exposure.

**C.** Propidium Iodide FACS profiles in SUM44 cells transfected six days earlier with siCON, or siFGFR1, and treated for 72 hours with 10ng/ml FGF2, 10nM 4OH-tamoxifen, 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% vs FGF2/4OH-T treated 13.4% ( $p=0.3$ , Student's T test); siFGFR1 transfected 7.2% vs 4.5% respectively ( $p=0.02$ ).



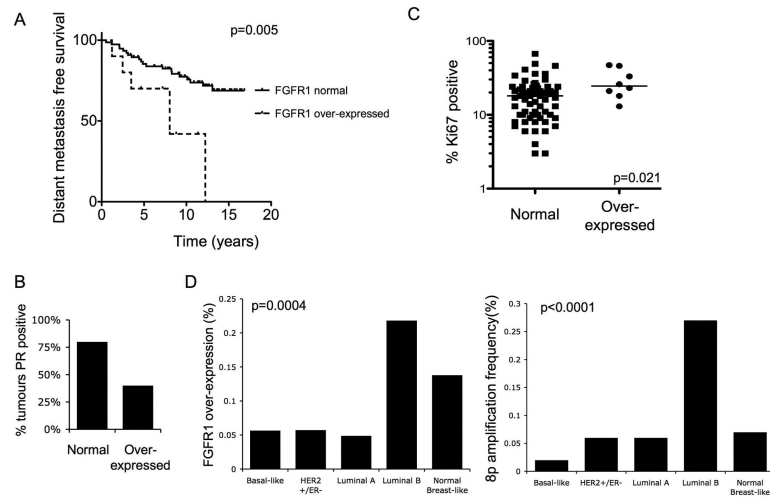
**Figure 5. Signalling in SUM44 cells is response to endocrine therapies**

**A.** Western blots of PR, ER, phosphorylated-ERK1/2, ERK1/2, CCND1, β-ACTIN, and FGFR1. SUM44 cell lysates treated for 24 hours prior to lysis with 100nM 4-OHT, 100nM ICI-182780, or no treatment (–), with or without 10ng/ml FGF2. Phosphorylated-AKT1 was not detected.

**B.** Western blots of phosphorylated-PLCγ1 (Tyr783), phosphorylated-AKT, phosphorylated-p90RSK (Thr359/Ser363), phosphorylated-ERK1/2, and β-ACTIN on SUM44 cell lysates treated for either 10 minutes or 24 hours with 10ng/ml FGF2 prior to lysis.

**C.** Quantitative RT-PCR analysis of cyclin D1 (*CCND1* - Top) and (*PR* - Bottom) expression in SUM44 cells treated with or without 10ng/ml FGF2 for 24 hours prior to RNA isolation, without (Black bars) or in the presence of 100nM ICI-182780 (Grey bars).

**D.** SUM44 cells were co-transfected with EREIItkLuc (ERE-luciferase reporter construct) and pCH110 (β-galactosidase reporter construct) and treated for 48 hours with 10ng/ml FGF2, or no treatment, with 100nM ICI-182780 as positive control. Luciferase activity was expressed relative to β-galactosidase activity. Error bars SEM of 3 repeats, p values Student's T-test.



**Figure 6. *FGFR1* over-expression is common in high risk ER positive breast cancer**

**A.** Kaplan-Meier curves of distant metastasis free survival from Guys series of ER positive tumours with *FGFR1* over-expression (n=10) versus normal *FGFR1* expression (76).

*FGFR1* over-expressing tumours have substantially worse survival (HR 7.4, 95% CI 1.8 to 30.5, p=0.0053, log rank test).

**B.** Proportion of tumours with progesterone receptor expression in the same cohort (all tumours were ER positive, p=0.03 Fisher's exact test).

**C.** Ki67 was assessed by IHC in the same cohort. *FGFR1* over-expressing tumours have higher Ki67 (P=0.021, Mann Whitney U Test). Of tumours with high proliferation (14% Ki67 as a surrogate for luminal B subtype(2)) 16% have *FGFR1* over-expression, compared to 3.5% of low proliferation cancers.

**D.** Left: Incidence of *FGFR1* over-expression in breast cancers according to intrinsic subtype (23% Luminal B over-express *FGFR1*). Right: Incidence of 8p11-12 amplification, defined by co-overexpression of neighbouring genes (Supplementary methods), according to intrinsic subtype (27% Luminal B have 8p11-12 amplification). Analysis of data on 295 invasive breast cancers from van de Vijver et al 2002(31). Statistical comparison across groups was with the Chi squared test.