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

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Supplementary methods and figure legends

Supplementary methods

Characterisation of cell lines

All cell lines were obtained from ATCC or Asterand and passaged in cell culture for less than 6 months, with the exception of the following lines. S68 was obtained from a non-commercial source. Array CGH characterisation is supplied as Supplementary Table 1. The original MDA-MB-134 was obtained directly from MD Anderson and was characterised in Reis-Filho et al 2006 (1).

Antibodies

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), β-ACTIN (sc-1616) (all Santa-Cruz Biotechnology, Santa Cruz, CA).

Analysis of KRAS mutation: A portion of the *KRAS* gene, including the region corresponding to amino acids 12 and 13, was amplified using primers for genomic DNA Forward 5'-AGGCCTGCTGAAAATGACTG-3' Reverse 5'-ATCAAAGAATGGTCCTGCAC-3' or cDNA Forward 5'-

AGGCCTGCTGAAAATGACTG-3'   Reverse   5'-TTGTTGGATCATATTCGTCCAC-3'
and directly sequenced with the corresponding PCR primers.

FGF2 ELISA: FGF2 expression was assayed using Elisa Duokit (DY233, R&D Systems) according to manufacturer's instructions. A standard curve of recombinant FGF2 (Sigma) in PBS was used to estimate FGF2 concentration.

Analysis of FGFR1 expression and 8p11-12 amplicon frequency in NKI series
Whole genome gene expression data was analysed on 295 tumours from van de Vijver et al 2002 (2). Tumours were classified into intrinsic subtypes according to the single sample predictor of Hu et al 2006 (3).

Analysis of FGFR1 over-expression: The mean of two probes reporting on *FGFR1* was taken, and the standard deviation of the data was estimated from the median absolute deviation of luminal A subtype tumours. A sample was considered to have *FGFR1* over-expression when level exceeded 3 standard deviations from the median, to identify samples with outlier over-expression.

Amplification of chromosomal region 8p11-12: We identified tumours with amplification of the locus of *FGFR1* by identifying tumours with coordinated over-expression of genes in the 8p11-12 amplicon. We selected genes whose expression had been shown to correlate with copy number (4): PROSC, RAB11FIP1 (Two probes), BRF2, ASH2L, ERLIN2 (Three probes), BAG4 (Two probes), LSM1, and DDHD2. To ensure independence from the *FGFR1* expression analysis, we excluded *FGFR1* probes from this analysis. A tumour was defined as being amplified if the expression of at least 10 of 12 probes had expression higher than the 3rd quartile. This cut-off divided the biphasic distribution (Supplementary Figure 5), and generated an overall frequency of 8p11-12 amplification (9.2%, 27/295) highly comparable with prior estimates of the frequency (5, 6). The data for different cut-offs

is supplied below: confirming that regardless of cut-off expression defined

amplification of 8p11-12 is characteristic of luminal B type cancers

Overexpression cut-off (of 12 probes)	Luminal A (n=123)	Luminal B (n=55)	Her2+ / ER- (n=35)	Basal (n=53)	Normal (n=29)	% amplified cancers that are luminal B
12	0 (0%)	7 (13%)	1 (3%)	0 (0%)	0 (0%)	88%
11	4 (3%)	12 (22%)	1 (3%)	0 (0%)	1 (3%)	67%
10	7 (6%)	15 (27%)	2 (6%)	1 (2%)	2 (7%)	56%
9	8 (7%)	18 (33%)	2 (6%)	1 (2%)	2 (7%)	58%
8	8 (7%)	19 (35%)	2 (6%)	3 (6%)	3 (10%)	54%
7	15 (12%)	21 (38%)	3 (9%)	4 (8%)	3 (10%)	46%
6	20 (16%)	24 (44%)	3 (9%)	5 (9%)	3 (10%)	44%

Supplementary Figure legends

Supplementary Figure 1. MDA-MB-134 has acquired a *KRAS* mutation.

A. Transfection of MDA-MB-134, obtained directly from the originating lab at MD Anderson, with four individual siRNA targeting FGFR1 with survival assessed at 6 days post transfection with Cell Titre-Glo® cell viability assay (Promega).

B. Sequencing of *KRAS* gene from MCF7 controls and MDA-MB-134 obtained from ATCC, with 35G>C heterozygous mutation in MDA-MB-134.

C. *FGFR1* expression assessed by real time PCR in MDA-MB-134 *KRAS* mutant cells transfected 72 hours earlier with siCON or siFGFR1.

Supplementary Figure 2. MDA-MB-134 *KRAS* mutant cells are dependent on FGFR signalling in the presence of MEK inhibitor.

A. MDA-MB-134 *KRAS* cells were serum starved for 24 hours in the presence of 10uM U0126, or no U0126, and treated with FGF2 1ng/ml for 10 minutes, 60 minutes, or no FGF2 (-) prior to cell lysis. MDA-MB-134 *KRAS* cells show substantial increased phosphorylation of ERK1/2 with FGF2 that is blocked by U0126. In the presence of U0126 FGF2 signals through AKT.

B. MDA-MB-134 K-RAS cells plated in 96 well plates were treated with indicated doses of U0126 in combination with FGF2 and survival assessed after 3 days exposure. Growth is expressed relative to DMSO only controls. Error bars SEM of three repeats. In this experiment we noted no evidence of growth inhibition by FGF2 as has been previously reported in this cell line (7).

C. MDA-MB-134 *KRAS* cells were serum starved for 24 hours in the presence of 10uM U0126, with or without 1μM PD173074, and treated with FGF2 1ng/ml for 10 minutes prior to lysis. Western blots of lystes blotted with indicated antibodies. Signalling in response to FGF2, in this setting, is blocked by PD173074.

Supplementary Figure 3. Array CGH profiles and signalling in *FGFR1* amplified cell lines.

A. arrayCGH profiles of chromosome 8 from MDA-MB-134 *KRAS*, SUM44, S68, and CAL120 with black arrow indicating genomic position of *FGFR1.*

B. Indicated cell lines were serum starved for 24 hours, and 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 AKT1-Ser473, phosphorylated ERK1/2-Thr202/Tyr204, phosphorylated RSK-Thr359/Ser363, phosphorylated PLCgamma1-Tyr783, ERK1/2 (9102), and β-ACTIN.

Supplementary Figure 4. CAL120 cells express autocrine FGF2.

A. Quantitative RT-PCR for *FGF2* mRNA in RNA from indicated cell lines (all *FGFR1* amplified with the exception of MCF7 andT47D) expressed relative to endogenous controls MRPL19 and βACTIN expression. CAL120 was the only cell line that expressed FGF2 mRNA at appreciable levels, at least 750 fold higher levels than any other cell line. We could not demonstrate appreciable levels of FGFR1 ligand expression in all other *FGFR1* amplified cell lines by quantitative PCR for FGF1 and FGF2, and whole genome expression profiling (data not shown).

B. Indicated cell lines were grown for 72 hours, before snap freezing of conditioned media. FGF2 concentration was assessed by ELISA. Concentration of FGF2 in non-conditioned media was 19 pg/ml, with no significant difference in conditioned media from SUM44 or T47D cells. In contrast, FGF2 concentration was elevated in CAL120 conditioned media 94pg/ml, $p < 0.0001$ Student's T-test, confirming autocrine production. Error bars SEM of three measurements.

Supplementary Figure 5. T47D cells expressing FGFR1 demonstrate resistance to endocrine therapies in response to FGF2.

A. T47D cells infected with control empty vector (T47D-EV) or FGFR1 expression vector (T47D-FGFR1, Figure 3B) were treated range of concentrations of 4-OHT in the presence of 10ng/ml FGF2, or with no FGF2. Survival was assessed after 6 days exposure, and expressed relative to cells treated with or without FGF2, respectively, in the absence of 4-OHT. FGF2 has only a minor non-significant effect on the sensitivity of T47D-EV cells to tamoxifen (p>0.05 at 100nm 4-OHT), whereas FGF2 has a substantial effect on the sensitivity of T47D-FGFR1 cells to 4-OHT (p<0.01 at 100nm 4-OHT).

B. T47D cells infected with control empty vector (T47D-EV) or FGFR1 expression vector (T47D-FGFR1) were deprived of oestrogen (Media with 10% DCC stripped serum with no E2 added) and grown for six days with or without FGF2 10ng/ml. Growth was expressed relative to E2 deprived cells without FGF2, Error bars SEM and p value Student's T Test.

Supplementary Figure 6. *FGFR1* is amplified and over-expressed in ER positive breast cancer.

A. Analysis of published Affymetrix U133A gene expression microarray data from Chin et al Cancer Cell 2007 (8). *ESR1* expression plotted against *FGFR1* expression from 90 tumours. Tumours divide into two groups of ER positive and negative along the X axis, and only ER positive tumours over-express *FGFR1*.

B. array CGH data from Chin et al Cancer Cell 2007 (8), displayed according to ER status. High level amplification of FGFR1 is found in ER positive breast cancers. The single ER negative tumour with high level amplification of *FGFR1* was PR positive. Tumours with CGH ratio greater than 1, ER and/or PR positive 11% (11/102) vs ER/PR negative 0% (0/43) p=0.036 Mann Whitney U Test.

References

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