Breast Cancer Res Treat (2008) 111:79–91
DOI 10.1007/s10549-007-9763-9

PRECLINICAL STUDY

Insulin receptor substrate-1 involvement in epidermal growth factor receptor and insulin-like growth factor receptor signalling: implication for Gefitinib ('Iressa') response and resistance

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Received: 6 December 2006 / Accepted: 13 September 2007 / Published online: 28 September 2007

Abstract Classically the insulin receptor substrate-1 (IRS-1) is an essential component of insulin-like growth factor type 1 receptor (IGF-IR) signalling, providing an interface between the receptor and key downstream signalling cascades. Here, however, we show that in tamoxifen-resistant MCF-7 (Tam-R) breast cancer cells, that are highly dependent on epidermal growth factor receptor (EGFR) for growth, IRS-1 can interact with EGFR and be preferentially phosphorylated on tyrosine (Y) 896, a Grb2 binding site. Indeed, phosphorylation of this site is greatly enhanced by exposure of these cells, and other EGFR-positive cell lines, to EGF. Importantly, while IGF-II promotes phosphorylation of IRS-1 on Y612, a PI3-K recruitment site, it has limited effect on Y896 phosphorylation in Tam-R cells. Furthermore, EGF and IGF-II co-treatment, reduces the ability of IGF-II to phosphorylate Y612, whilst maintaining Y896 phosphorylation, suggesting that the EGFR is the dominant recruiter of IRS-1 in this cell line. Significantly, challenge of Tam-R cells with the EGFR-selective tyrosine kinase inhibitor gefitinib, for 7 days, reduces IRS-1/EGFR association and IRS-1 Y896 phosphorylation, while promoting IRS-1/IGF-IR association and IRS-1 Y612 phosphorylation. Furthermore, gefitinib significantly enhances IGF-II-mediated phosphorylation of IRS-1 Y612 and AKT in Tam-R cells. Importantly, induction of this pathway by gefitinib can be abrogated by inhibition/downregulation of the IGF-IR. Our data would therefore suggest a novel association exists between the EGFR and IRS-1 in several EGFR-positive cancer cell lines. This association acts to promote phosphorylation of IRS-1 at Y896 and drive MAPK signalling whilst preventing recruitment of IRS-1 by the IGF-IR and inhibiting signalling via this receptor. Treatment with gefitinib alters the dynamics of this system, promoting IGF-IR signalling, the dominant gefitinib-resistant growth regulatory pathway in Tam-R cells, thus, potentially limiting its efficacy.

Keywords IRS-1 · EGFR · IGF-1R · Gefitinib · Tamoxifen resistance · MCF-7

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Introduction

The epidermal growth factor receptor (EGFR), a member of the c-erbB receptor tyrosine kinase family, has been implicated in the aetiology of a wide range of epithelial cancers including breast, prostate, non-small cell lung and colon [1–3]. Aberrant activation of EGFR can prime multiple downstream signalling cascades, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K) pathways, which mediate key mechanisms underlying tumour growth and progression, providing a strong rationale to target this receptor [4, 5]. Two major classes of agent have been developed to inhibit EGFR activity, monoclonal antibodies, such as cetuximab (Erbitux, C225), which bind the extracellular ligand-binding domain of the receptor and small molecule tyrosine kinase inhibitors, such as the quinazolone derivatives gefitinib (Iressa, ZD1839) and erlotinib (Tarceva, OSI-774), which competitively block binding of adenosine triphosphate to the receptors tyrosine kinase domain [6]. These anti-EGFR therapies have been shown to have potent

anti-tumour activity in the preclinical and clinical setting both as monotherapies [6–9] and when utilised in combination with conventional radiation and chemotherapies where they have been shown to enhance the effects of cytotoxic agents [10–13]. However, despite this clear therapeutic promise clinical trials have disappointingly revealed evidence of primary/de novo and acquired resistance to these EGFR inhibitors. A range of possible resistance mechanisms have been identified in both preclinical and clinical studies and include receptor mutation [14–16], loss of downstream effector components [17] and activation of alternative oncogenic signalling pathways [18–21].

A candidate resistance mechanism to anti-c-erbB receptor therapy in a number of cancer types is the insulin-like growth factor receptor (IGF-IR) signalling pathway. The IGF-IR is a member of the type II receptor tyrosine kinase family, which also includes the insulin receptor [22] and has been linked to disease progression and recurrence in clinical breast cancer [23, 24]. Ligand binding of insulin, insulin-like growth factor 1 or II (IGF-I or IGF-II) leads to receptor autophosphorylation and subsequent phosphorylation of substrate proteins such as the insulin receptor substrate-I (IRS-1; 25). IRS-1 is a major substrate for IGF-IR and can be phosphorylated at a number of tyrosine residues, in particular tyrosine (Y) 896, a growth factor receptor-bound protein 2 (Grb2) binding site leading to recruitment of the MAPK signalling pathway, and Y612 which is a binding site for the p85 subunit of PI3K, key pathways contributing to the oncogenic potential of IGF-IR [25–27]. IGF-IR signalling via the PI3-K/Akt pathway has been shown to mediate resistance to the anti-c-erbB2 monoclonal antibody trastuzumab in SKBR3 and c-erbB2-transfected MCF-7 breast cancer cell lines [28, 29]. More recently, increased IGF-IR signalling activity has also been implicated in the development of resistance to the selective EGFR tyrosine kinase inhibitor AG1478 in glioblastoma cells [19] and to the anti-EGFR monoclonal antibody 225 in the DiFi human colorectal cancer cell line [18]. We have also recently reported that tamoxifen-resistant MCF-7 (Tam-R) breast cancer cells that have acquired resistance to the selective EGFR tyrosine kinase inhibitor gefitinib demonstrate elevated levels of phosphorylated IGF-IR and an increased sensitivity to growth inhibition by the IGF-IR inhibitor, AG1024 [20]. Similar findings have also been reported in SK-BR-3 breast cancer cells and the EGFR-positive DU145 prostate cancer cell line [20–21].

In the present study, we have examined potential mechanisms of cross-talk between EGFR and components of the IGF-IR signalling pathway, in particular IRS-1, in several EGFR-positive human cancer cell lines and what role this cross-talk may have on early response to gefitinib challenge. We demonstrate that a novel association between EGFR and IRS-1 exists in breast, prostate and lung cancer cell lines, which may serve to reduce association of IRS-1 and IGF-IR and inhibit signalling via this receptor. We further show that, in Tam-R MCF-7 cells, EGFR inhibition can promote IGF-IR signalling through the re-establishment of its links with IRS-1, providing a potential survival/resistance signal and that, as a consequence, co-targeting of EGFR and IGF-IR in Tam-R cells can generate a more effective inhibition of cell growth compared to gefitinib treatment alone.

Materials and methods

Cell culture

The tamoxifen-resistant cell line (Tam-R) was developed by continually exposing WT-MCF-7 breast cancer cells to 4-hydroxytamoxifen (100 nM) over a period of 6 months [30]. The WT-MCF-7 and Tam-R cells were grown in phenol-red-free RPMI medium containing 5% charcoal-stripped steroid-depleted fetal calf serum (FCS), penicillin–streptomycin (10 IU/ml–10 μg/ml), fungizone (2.5 μg/ml) and glutamine (4 mM). The medium for Tam-R cells was also supplemented with 4-hydroxytamoxifen (100 nM in ethanol). The T47D breast and LNCaP prostate cancer cells were grown in the same growth medium again but containing 10% FCS. DU145 prostate and A549 non-small cell lung carcinoma (NSCLC) cells were routinely cultured in Dulbecco's Minimum Eagle's Medium (DMEM) supplemented with 10% FCS. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Experimental procedures

Western blotting studies

Tamoxifen-resistant, WT-MCF-7, T47D, DU145 and LNCaP cell lines were grown for 4 days to allow the cells to achieve approximately 70% confluence prior to transfer into phenol-red/steroid-and serum free DCCM (Biosynergy Europe, Cambridge, UK) for 24 h. A549 cells were similarly grown to 70% confluence but were then transferred to DCCM supplemented with 0.5% FCS for 24 h. The cells were then lysed to measure basal protein expression. To examine the effects of pharmacological agents, cells were lysed following a further incubation in either DCCM or phenol-red-free RPMI medium supplemented with either 5% FCS for up to 7 days, IGF-II (10–100 ng/ml in 10 mM acetic acid/0.1% Bovine Serum Albumen; R&D Systems, Abingdon, UK) for 5 min, epidermal growth factor [EGF; 10 ng/ml in phosphate buffered saline (PBS)] for 5 min,

the specific IGF-IR tyrosine kinase inhibitor 4-anilino-5-bromo-2-[4-(2-hydroxy-3-(N,N-dimethylamino) propoxy) anilino] pyrimidine (ABDP; 1 μM in DMSO, AstraZeneca, Macclesfield, UK [31]) for 7 days and the selective EGFR tyrosine kinase inhibitor gefitinib (1 μM in ethanol, kind gifts from AstraZeneca, Macclesfield, UK) for up to 7 days or a combination of these treatments. Controls in all cases were incubated for the same periods of time with or without the appropriate vehicle. All experiments were performed at least three times.

Growth studies

Tam-R cells were grown continuously over a period of 23 weeks in phenol-red-free RPMI medium containing charcoal-stripped 5% FCS supplemented with either gefitinib alone (1 μM), ABDP alone (0.25 μM) or gefitinib and ABDP in combination. Controls were incubated for the same period of time with the appropriate vehicle. Cell population growth was evaluated by the number of passages the cells had undergone over this time period. Cells were split 1:10 at each passage.

Small interfering RNA transfection

Dharmacon SMARTpool siRNA reagents (four pooled SMART selection-designed siRNAs) specific for either IGF-IR or EGFR were diluted to a working stock concentration of 20 μM in sterile Dharmacon RNAse free 1× siRNA buffer. SMARTpool siRNA were then mixed with Dharmafect 1 transfection reagent at a ratio of 10 μl siRNA: 0.75 μl reagent and incubated at room temperature for 20 min. The transfection reagent mix was added to the appropriate dish of Tam-R cells, which were maintained in antibiotic-free phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS, to give a final siRNA concentration of 100 nM per dish. Control experiments consisted of transfection with the non-targeting siRNA scrambled control (100 nM), medium only (non-transfected cells), or Dharmafect 1 reagent only (lipid). All experimental arms were set up in duplicate. Cells were then incubated for a period of 4 days prior to treatment with either IGF-II (100 ng/ml in 10 mM acetic acid/0.1% BSA) or vehicle alone for 5 min. The cells were then lysed and protein extracts were subjected to Western blot analysis.

Cell lysis

Cells were washed three times with PBS and cell lysis was performed as previously described [30]. Briefly cells were lysed using 200 μl ice cold lysis buffer (50 mM Tris, pH 7.5, 5 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′N′-tetraacetic acid, 150 mM NaCl and 1% triton X 100) containing protease inhibitors (2 mM sodium othovanadate, 200 mM sodium flouride, 1 mM phenylmethylsulfonyl flouride, 20 μM phenylarsine, 10 mM sodium molybdate, 10 μg/ml leupeptin and 10 μg/ml aprotinin. The cellular contents were transferred to eppendorf tubes and clarified by centrifugation at 13,000 rpm for 15 min at 4°C and supernatant aliquots were stored at –20°C until required. Total protein concentrations were determined using the DC BioRad protein assay kit (BioRad Labs Ltd, Hemel Hempstead, UK).

Immunoprecipitation

Cell lysates containing 1 mg protein were immunoprecipitated using 1 μg specific antibody and tubes were incubated on ice for 1 h. Thirty microlitres of protein A agarose (Insight Biotechnology Ltd, Wembley, UK) was added to the mixture, and the tubes were placed onto a rotary mixer at 4°C for a further 2 h. The immune complex was centrifuged at 3,000 rpm at 4°C for 5 min and washed with ice cold lysis buffer. This procedure was repeated twice and the resultant pellet resuspended in 20 μl 2× Laemelli sample loading buffer containing fresh 0.01 M dithiothreitol. Samples were boiled at 100°C for 5 min to release the bound proteins, centrifuged at 13,000 rpm at 4°C for 1 min to pellet the beads prior to gel loading.

Western blotting

Protein samples from either total cell lysates (20–100 μg) or following immunoprecipitation were subjected to electrophoresis separation on a 7.5% polyacrylamide gel and then trans-blotted onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Blots were blocked at room temperature for 1 h in 5% w/v non-fat dried milk made up in Tris-buffered saline (TBS)-Tween 20 (0.05%) and then incubated for a minimum of 2 h in primary antibody diluted 1/1,000 in 5% Western Blocking Reagent/TBS-Tween 20 (0.05%) (Roche Diagnostics, Mannheim, Germany). The membranes were washed three times in TBS-Tween 20 (0.05%) and then incubated for 1 h with secondary IgG horse radish peroxidase labelled donkey anti-rabbit antibody (Amersham, UK), diluted 1/10,000 in 1% w/v non-fat dried milk made up in TBS-Tween 20 (0.05% v/v). Detection was performed using West Dura long duration and femto sensitive chemiluminescent detection reagents (Pierce and Warriner Ltd, Cheshire, UK). Antibodies used were directed against total EGFR (SC-03),

total erbB2 (SC-284), total IGF-IR (SC-712), total IRS-1 (SC-72000), Grb2 (SC-255), rabbit IgG (SC-2027), phosphorylated tyrosine (pY20, SC-508) (Insight Biotechnology Ltd, Wembley, UK), phosphorylated EGFR (pY1068), total AKT (9272), phosphorylated AKT (pS473, 9271), total ERK1/2 (9102) and phosphorylated ERK1/2 (pT202/pY204, 9101) (New England Biolabs, Hertfordshire, UK), phosphorylated IRS-1 (Y612 and Y896; Biosource International, USA), total IRS-2 (Upstate Ltd, Dundee, UK), β-actin (Sigma, UK) and phosphorylated IGF-IR (Y1316); a kind gift from AstraZeneca, Macclesfield, UK.

Results

EGFR and IGF-IR signalling activity in tamoxifen-resistant MCF-7 cells

Under basal growth conditions (e.g. in the absence of exogenous growth factors), Western blotting analysis revealed that Tam-R cells expressed detectable levels of total and activated EGFR and ERK1/2 as previously reported [30]. Similarly, expression of total and activated IGF-IR and AKT was also observed in this cell line [32, 33] (Fig. 1a). Assessment of the basal IRS-1 phosphorylation profile revealed high levels of phosphorylation on residue Y896 but low expression of IRS-1 Y612 phosphorylation in this cell line (Fig. 1a).

IRS-1 associates with EGFR in Tam-R cells

To examine IRS-1 phosphorylation in Tam-R cells we investigated the effects of ligand stimulation. Western blotting revealed that IGF-II promoted substantial phosphorylation of IGF-IR, a small increase in EGFR phosphorylation levels and activation of AKT in the Tam-R cell line (Fig. 1a). IGF-II also promoted a marked phosphorylation of IRS-1 at Y612 (Fig. 1a, b). IGF-II induced only a small phosphorylation of IRS-1 at Y896 in these cells (Fig. 1a, b). EGF stimulation resulted in elevated phosphorylation of EGFR, ERK1/2 and AKT but was without effect on IGF-IR phosphorylation in the Tam-R cell line (Fig. 1a). Interestingly, in contrast to IGF-II stimulation, EGF promoted substantial phosphorylation of IRS-1 at Y896 with no visible effect on IRS-1 Y612 levels being observed in this cell line (Fig. 1a, b). Neither ligand influenced total expression levels of these proteins.

Immunoprecipitation/Western blotting (IP/WB) analysis was performed to examine whether IRS-1 phosphorylation at Y896 resulted from an interaction with EGFR. Preliminary studies confirmed the specificity of the anti-IRS-1 and anti-EGFR antibodies used in these studies (Fig. 2a).

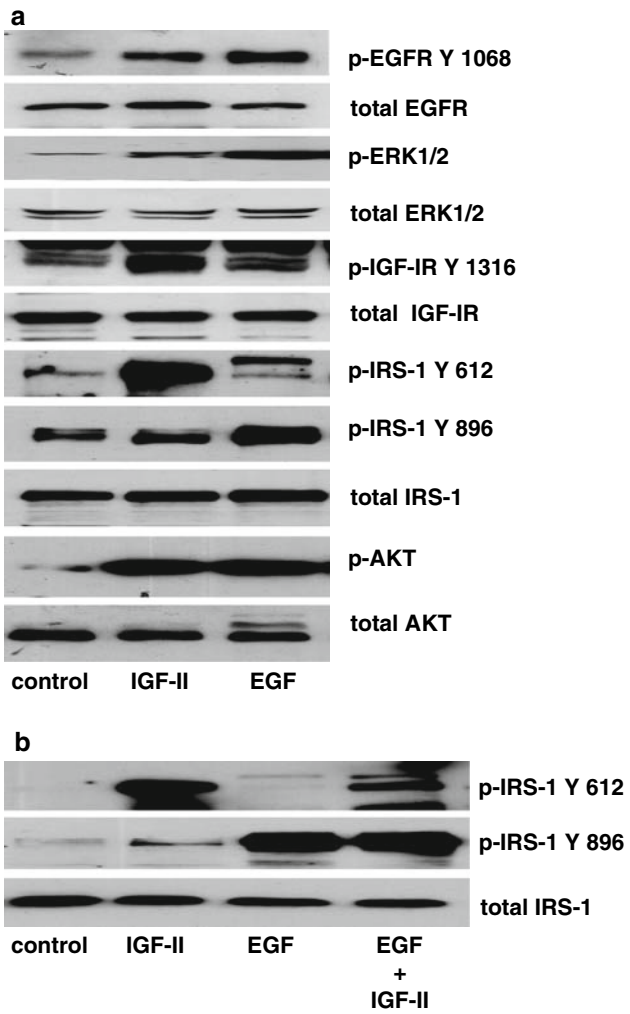

Fig. 1 Western analysis of (**a**) phosphorylated and total EGFR, ERK1/2, IGF-IR, IRS-1 and AKT expression following incubation of Tam-R cells in serum-free DCCM supplemented with either EGF (10 ng/ml, 5 min), IGF-II (100 ng/ml, 5 min) or appropriate vehicle control and (**b**) phosphorylated IRS-1 (Y896 and Y612) and total IRS-1 expression in Tam-R cells incubated in serum-free DCCM supplemented with either IGF-II (100 ng/ml), EGF (10 ng/ml), a combination of the two agents or appropriate vehicle control. Data are representative of at least three separate experiments

IP/WB analysis revealed that EGF promoted the ability of EGFR and IRS-1 to associate in Tam-R cells with a resultant increased level of phosphorylation of IRS-1 at Y896 and enhanced recruitment of Grb2 to IRS-1 (Fig. 2b–d). There was no evidence of an association between EGFR with IGF-1R either under basal or EGF-treated conditions in this cell line (Fig. 2c).

EGFR is the dominant recruiter of IRS-1 in Tam-R cells

Western blotting analysis revealed that co-treatment of Tam-R cells with EGF and IGF-II resulted in

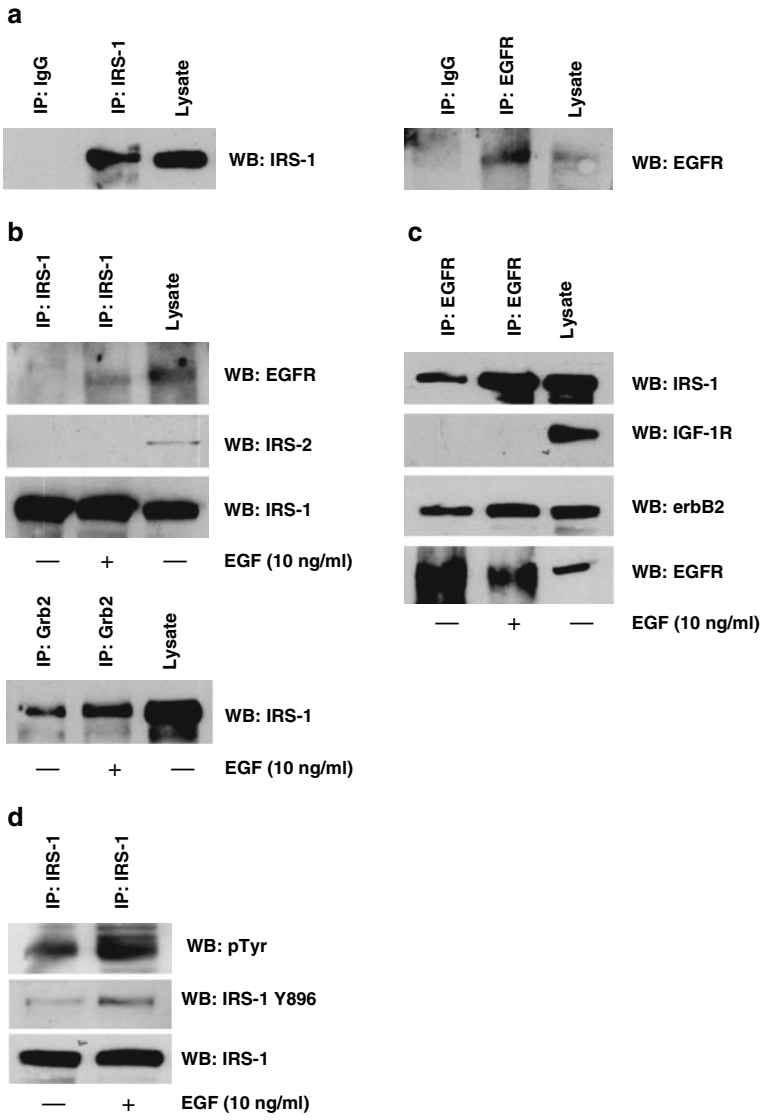

Fig. 2 Western analysis of (**a**) total IRS-1 and EGFR expression following immunoprecipitation with either rabbit IgG (negative control), total IRS-1 or EGFR antibody in Tam-R cells incubated in serum-free DCCM, (**b**) total EGFR, IRS-2 and IRS-1 expression following immunoprecipitation with either total IRS-1 or Grb2 antibody in Tam-R cells incubated in serum-free DCCM supplemented with either EGF (10 ng/ml) or vehicle control, (**c**) total IRS-1, IGF-1R, erbB2 and EGFR expression following immunoprecipitation with total EGFR antibody in Tam-R cells incubated in serum-free DCCM supplemented with either EGF (10 ng/ml) or vehicle control and (**d**) phosphorylated tyrosine residue and total and phosphorylated IRS-1 (Y896) expression following immunoprecipitation with total IRS-1 antibody in Tam-R cells incubated in serum-free DCCM supplemented with either EGF (10 ng/ml) or vehicle control. Data are representative of at least three separate experiments

phosphorylation of IRS-1 at both Y612 and Y896, however, levels of Y612 phosphorylation were lower following combined treatment with the two ligands compared with IGF-II stimulation alone, suggesting a reduced ability of IGF-II to phosphorylate IRS-1 at this tyrosine residue (Fig. 1b). Levels of IRS-1 Y896 phosphorylation, however, were found to be the same in both EGF stimulated and EGF/IGF-II stimulated cells whilst total levels of IRS-1 remained unchanged for all treatments studied (Fig. 1b).

EGFR blockade promotes association of IRS-1 with IGF-IR in Tam-R cells

Basal EGFR, ERK1/2 and IRS-1 Y896 phosphorylation levels were markedly inhibited following treatment of Tam-R cells with gefitinib (1 μM) for 7 days (Fig. 3a). However, an increase in basal levels of IRS-1 Y612 and AKT phosphorylation was also observed as a result of this treatment regime (Fig. 3a). Total protein levels remained unchanged for all treatment groups. Similar findings to those for gefitinib were also observed following the silencing of the EGFR gene using targeted siRNA technology. The siRNA selectively down-regulated total EGFR protein expression, with total levels of all other proteins remaining unchanged (Fig. 3a). Phosphorylated EGFR, ERK1/2, AKT and IRS-1 Y896 levels were reduced quite substantially 4 days post transfection, whereas, there was an increase in basal levels of IRS-1 Y612 phosphorylation levels (Fig. 3a).

Immunoprecipitation/Western blotting analysis revealed that a 7 day gefitinib challenge (1 μM) of Tam-R cells resulted in a reduced association of IRS-1 with both EGFR and Grb2 alongside an increased association of IRS-1 with IGF-IR (Fig. 3b). Total levels of IRS-1 remained the same

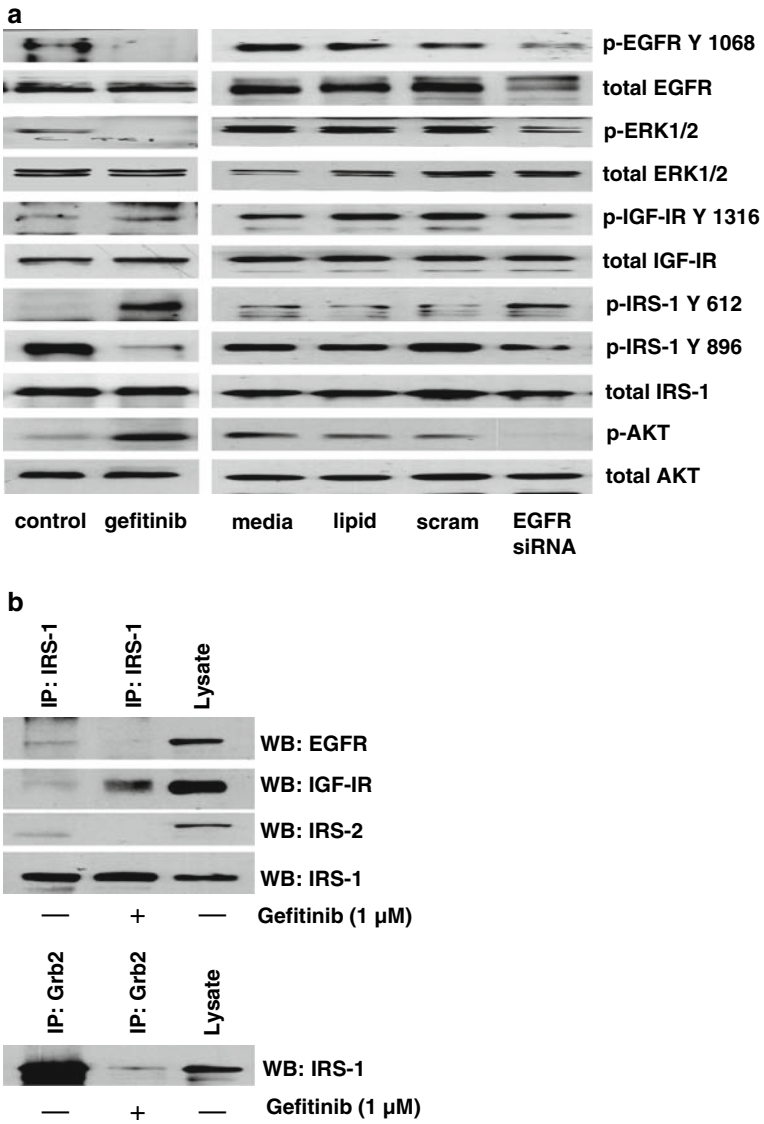

Fig. 3 (**a**) Western analysis of phosphorylated and total EGFR, ERK1/2, IGF-IR, IRS-1 and AKT expression following incubation of Tam-R cells in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 7 days supplemented with; *left panel*: either gefitinib (1 μM) or vehicle control, *right panel*: either cell culture medium (media), Dharmafect 1 transfection reagent alone (lipid), Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (scrambled, 100 nM) or Dharmafect 1 transfection reagent and EGFR siRNA mix (100 nM) for 4 days, (**b**) total EGFR, IGF-IR, IRS-2 and IRS-1 protein expression following immunoprecipitation with either total IRS-1 or Grb2 antibody in Tam-R cells incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 7 days supplemented with either gefitinib (1 μM) or vehicle control. Data are representative of at least three separate experiments

both in the absence and presence of gefitinib and expression of IRS-2 could not be detected demonstrating the selectivity of the anti-IRS-1 antibody used in these studies (Fig. 3b).

A time course study revealed that the increase in IRS-1 Y612 phosphorylation occurred as early as 1 h post gefitinib treatment, whereas, AKT activity was inhibited up to day 4 (mirroring results observed for siRNA). An increase in AKT activity was only observed following 7 days treatment with the EGFR inhibitor (Fig. 4a).

Co-targeting of EGFR and IGF-IR potently inhibits growth of Tam-R cells

Stimulation of Tam-R cells with increasing concentrations of IGF-II (3–100 ng/ml) in the absence of gefitinib promoted phosphorylation of IRS-1 at Y612 and AKT only at the highest concentration used (100 ng/ml) whilst no effect on total expression levels of these proteins was observed (Fig. 4b). However, following a 7-day treatment with gefitinib (1 μM), Tam-R cells demonstrated an increased sensitivity to IGF-II stimulation with phosphorylation of IRS-1 at Y612 and AKT being observed in response to both 30 and 100 ng/ml of the ligand (Fig. 4b). Similar findings were also observed with IGF-1 stimulation of Tam-R cells (data not shown).

The gefitinib-dependent enhancement of IGF-II-mediated IRS-1 Y612 and AKT phosphorylation could be reversed by treatment of the Tam-R cells with the specific IGF-IR inhibitor ABDP (1 μM) in combination with gefitinib (1 μM) (Fig. 5). In addition, phosphorylation of IRS-1 Y896, EGFR Y1068 and ERK1/2 levels following IGF-II stimulation were inhibited further in the presence of ABDP and gefitinib compared to gefitinib alone (Fig. 5). ABDP alone reduced phosphorylation of IGF-1R, IRS-1 Y612 and

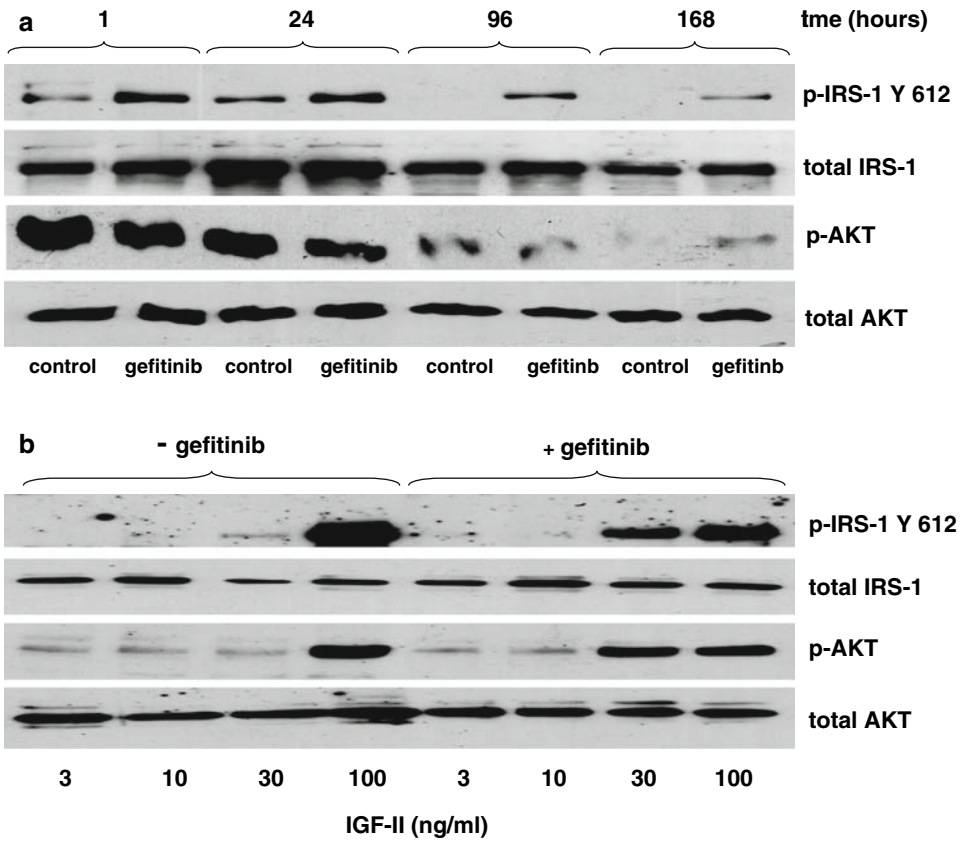

Fig. 4 Western analysis of phosphorylated IRS-1 (Y612), total IRS-1 and phosphorylated and total AKT protein expression following incubation of Tam-R cells in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with (**a**) either gefitinib (1 μM) or vehicle control for 1, 24, 96 and 168 h and (**b**) either gefitinib (1 μM) or vehicle control for 7 days and subsequently challenged with increasing concentrations of IGF-II (3–100 ng/ml, 5 min). Data are representative of at least three separate experiments

Y896, EGFR, AKT and ERK1/2 but not as effectively as the combination treatment. Total protein levels remained unchanged for all treatment groups. Similarly, the selective knockdown of both EGFR and IGF-IR protein expression using siRNA technology in Tam-R cells effectively prevented the enhanced IGF-II-mediated IRS-1 Y612 and AKT phosphorylation observed following transfection with siRNA targeting EGFR alone (Fig. 5). However, the further reduction in IRS-1 Y896, EGFR Y1068 and ERK1/2 phosphorylation levels seen with gefitinib and ABDP in combination was not so apparent following siRNA co-targeting of these receptors possibly due to the potent action of EGFR siRNA alone in this study (Fig. 5). IGF-1R siRNA alone reduced IGF-1R, IRS-1 Y612, AKT and ERK1/2 phosphorylation but was not as effective as ABDP in reducing EGFR and IRS-1 Y896 phosphorylation levels (Fig. 5).

To examine what role IGF-1R signalling may play in the development of gefitinib resistance we investigated the effect of long-term single and combination treatments on Tam-R cell growth. Both gefitinib and ADBP inhibited Tam-R cell growth as single agents, however, these growth inhibitory effects were only transient, lasting 6 weeks for ABDP and 15 weeks for gefitinib (Fig. 6). By 20 weeks, there was evidence of cell regrowth in both the gefitinib- and ADBP-treated Tam-R cells (Fig. 6). In Tam-R cells exposed to the combination of gefitinib and ADBP there was potent growth inhibition which was maintained through to week 19, by which time there was complete cell loss (Fig. 6).

EGFR recruits IRS-1 in a range of EGFR-positive cancer cell lines

Under basal growth conditions, phosphorylation of IRS-1 at Y896 was greater in Tam-R compared to WT-MCF-7 cells, however, treatment with EGF (10 ng/ml) increased Y896 phosphorylation of IRS-1 to a similar degree in both cell lines (Fig. 7a). Treatment of T47D, LNCaP, DU145 and A549 cancer cells with EGF also dramatically increased phosphorylayion levels of IRS-1 at Y896, mirroring results seen for Tam-R and WT-MCF-7 cells (Fig. 7b). Treatment of T47D, LNCaP, DU145 and A549 cancer cell lines with gefitinib (1 μM) for 7 days, revealed that basal phosphorylation levels of IRS-1 Y896 were inhibited in all cell lines studied again mirroring results seen for Tam-R cells (Fig. 7c). Gefitinib increased IRS-1 Y612 phosphorylation levels only in the Tam-R and LNCaP cell lines with no significant effect of this inhibitor being observed in the other three cancer cell lines studied (Fig. 7c). Total protein IRS-1 levels were found to be

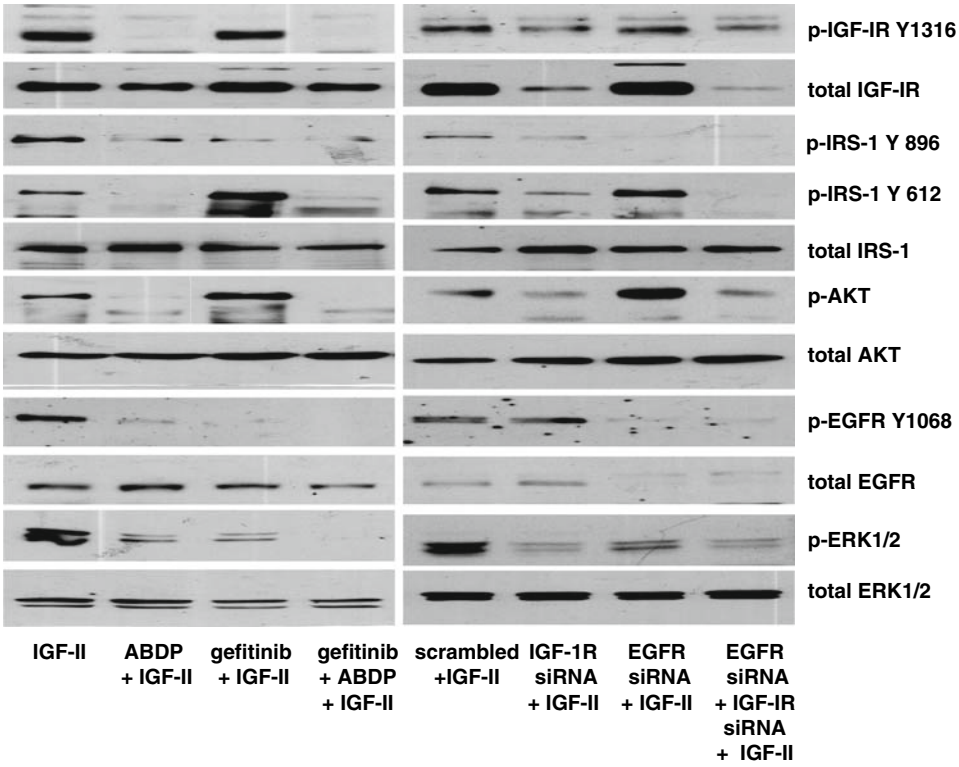

Fig. 5 Western analysis of total and phosphorylated IGF-IR, IRS-1, AKT, EGFR and ERK1/2 protein expression following incubation of Tam-R cells in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with; *left panel*: either ABDP or gefitinib for 4 days (1 μM), gefitinib (1 μM) in combination with ABDP for 4 days (1 μM) or appropriate vehicle control and subsequently challenged with either IGF-II (100 ng/ml) for 5 min, *right panel*: either Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (scrambled, 100 nM), Dharmafect 1 transfection reagent and IGF-1R siRNA mix (100 nM), Dharmafect 1 transfection reagent and EGFR siRNA mix (100 nM) or Dharmafect 1 transfection reagent, EGFR siRNA (100 nM) and IGF-IR siRNA mix (100 nM) for 4 days and subsequently challenged with either IGF-II (100 ng/ml) for 5 min. Data are representative of at least three separate experiments

differentially expressed among the cancer cell types with LNCaPs expressing the least amount of IRS-1, however, there was no effect of any of the treatments on total protein expression in any of the cell lines studied (Fig. 7a–c).

Discussion

We have recently demonstrated that an EGFR-positive tamoxifen-resistant MCF-7 breast cancer cell line (Tam-R) is initially growth inhibited by the selective EGFR tyrosine kinase inhibitor gefitinib, however, following prolonged exposure to this agent (4–6 months) the cells start to regrow due to acquisition of resistance [20]. We have gone on to show that this dual tamoxifen/gefitinib-resistant growth is mediated by signalling via the IGF-IR and PI3K/AKT and protein kinase C delta (PKCδ) pathways [20]. Similarly, we have reported that growth of a gefitinib-resistant DU145 prostate cancer cell line is also dependent on IGF-IR/AKT/PKCδ signalling activity. Furthermore, the IGF-IR has also been implicated in gefitinib resistance in SK-BR-3 breast cancer cells [21]. In the present study, we have examined interactions between EGFR and IRS-1 and whether the switch to utilising the IGF-IR signalling pathway is an early response to gefitinib treatment in our Tam-R cell line. We have also evaluated whether such a response to gefitinib is unique to this cell line by extending the study to incorporate other EGFR-positive cancer cell lines.

Initial characterisation of our Tam-R cell line under basal growth conditions confirmed previous findings with expression and activation of EGFR, IGF-IR and associated downstream signalling elements MAPK and AKT being evident [30, 32, 33]. We also found that these cells expressed high levels of IRS-1, a key substrate of the insulin and IGF-type 1 receptors. Interestingly, examination of the phosphorylation profiles of IRS-1 in this cell line revealed differential expression of two tyrosine phosphorylated forms of this adaptor protein. IRS-1 can be phosphorylated at multiple tyrosine residues following association with IR/IGF-IR; two key residues which when phosphorylated play a central role in recruitment of

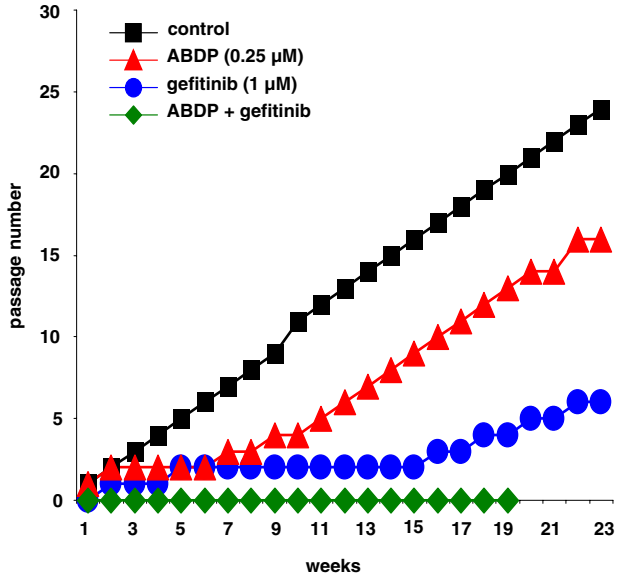

Fig. 6 The effects of gefitinib (1 μM) alone, ABDP (0.25 μM), the two agents in combination or vehicle control on the basal growth of Tam-R cells over a 23-week period. Growth is represented by the number of passages (cells split 1:10) the cells have undergone during this time period

important downstream signal transduction cascades being Y612 and Y896. Phosphorylation of IRS-1 at Y612 has been shown to act as a docking site for the p85 regulatory subunit of PI3-K which when activated serves to drive AKT activity, whereas, Y896 phosphorylation of this adaptor protein acts as a recruitment site for the adaptor protein Grb2 which is involved in triggering the MAPK signalling pathway [26, 27]. Treatment of Tam-R cells with IGF-II promoted predominantly Y612 phosphorylation of IRS-1, with only a small increase in IRS-1 Y896 phosphorylation being observed in response to this ligand. In contrast, EGF caused a sharp increase in Y896 phosphorylated IRS-1 levels whilst having no effect on IRS Y612 levels in this cell line. These findings suggest that firstly, IRS-1 Y612 phosphorylation appears to be solely under the regulation of the IGF-IR in Tam-R cells, thus, IGF-IR signals primarily through PI3K/AKT pathway in these cells. Secondly, EGFR appears to utilise IRS-1 as part of its mechanism to engage the MAPK signalling cascade in this same cell line, which was further supported by immunoprecipitation/Western blotting studies, which revealed that EGF-induced phosphorylation of IRS-1 at Y896, resulted from an association of EGFR with IRS-1 and recruitment of Grb2 by IRS-1. Such an association could reflect either a

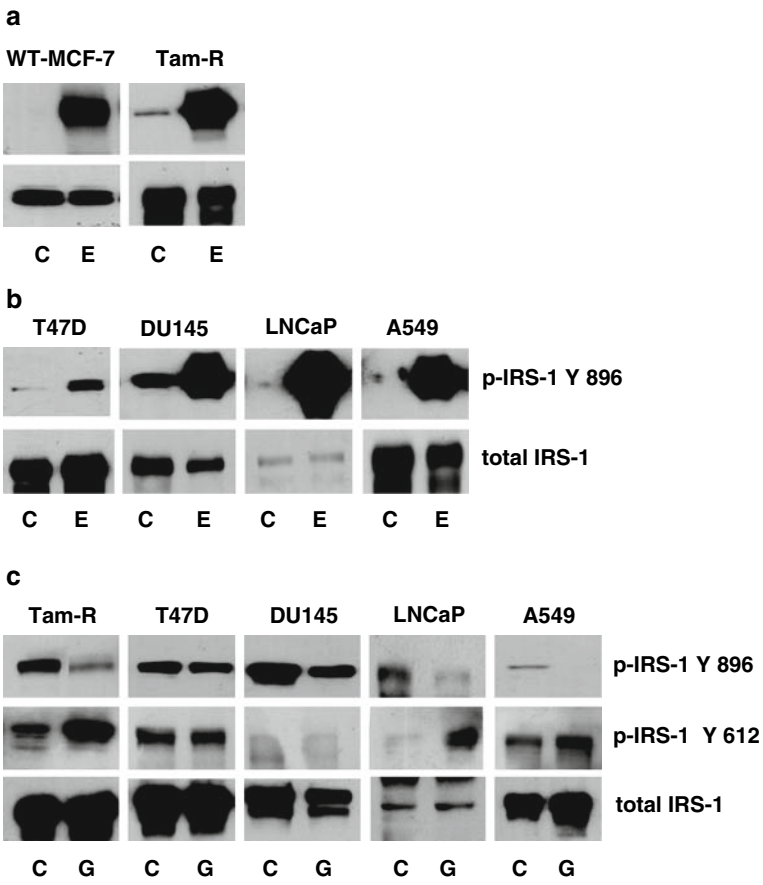

Fig. 7 Western analysis of total and phosphorylated IRS-1 protein expression following incubation of (**a**) WT-MCF-7 and Tam-R cells in serum-free DCCM supplemented with either EGF (*E*; 10 ng/ml) or appropriate vehicle control (*C*) for 5 min, (**b**) T47D, DU145 and LNCaP cancer cells in serum-free DCCM and A549 cancer cells in DCCM containing 0.5% FCS, supplemented with either EGF (*E*; 10 ng/ml) or appropriate vehicle control (*C*) for 5 min and (**c**) Tam-R, T47D, DU145 and LNCaP cancer cells in serum-free DCCM and A549 cancer cells in DCCM containing 0.5% FCS, supplemented with either gefitinib (*G*; 1 μM) or vehicle control (*C*). Data are representative of three separate experiments

direct physical interaction or the formation of a signalling complex with secondary proteins acting as a bridge between the two proteins. This EGFR/IRS-1 complex does not incorporate IGF-1R as we found that EGFR and IGF-1R do not associate in this cell line following immunoprecipitation/Western blotting studies, confirming our previous findings [32]. The small increase in IRS-1 Y896 phosphorylation in response to IGF-II may indicate the ability of IGF-II to activate EGFR, a mechanism we have previously demonstrated to be active in Tam-R cells [32].

The ability of EGFR to recruit IRS-1 is a novel signalling phenomenon that has not previously been described to date in breast cancer cells. However, we have found that such a phenomenon is not unique to tamoxifen-resistant MCF-7 breast cancer cells as EGF can also promote phosphorylation of IRS-1 at Y896 in a range of EGFR-positive cancer cell lines, T47D breast cancer cells, DU145 and LNCaP prostate cancer cells and A549 NSCLC cells. Interestingly, we also have evidence that EGF can promote phosphorylation of IRS-1 at Y896 in the parental WT-MCF-7 cells, however, this cross-talk mechanism is more prominent in Tam-R cells, particularly under basal growth conditions, probably as a consequence of the higher expression levels of EGFR in Tam-R cells [30]. EGF-dependent IRS-1 phosphorylation has also been reported in human epidermoid carcinoma A431 cells and in primary cultures of rat hepatocytes [34, 35]. However, it should also be noted that EGF is without effect on tyrosine phosphorylation of IRS-1 in 3T3-L1 adipocytes transfected with EGFR [36]. It is not entirely surprising that EGFR can recruit IRS-1. A potential interaction between EGFR and IRS-1 has been predicted from the binding of peptides, representing the physical sites of EGFR tyrosine phosphorylation, to protein microarrays comprising all Src homology 2 and phosphotyrosine binding domains encoded in the human genome [37]. Furthermore, the phosphorylated NPXY motifs in activated insulin and IGF-IR receptors to which the phosphotyrosine binding domains of IRS molecules bind are also present in the C-terminus region of EGFR [38]. Indeed, the presence of all three of these NPXY motifs found in EGFR were found to be indispensable for IRS-1 to be tyrosine phosphorylated in response to EGF in EGFR-transfected Chinese hamster ovary cells [34].

The EGFR appears to be the dominant recruiter of IRS-1 in Tam-R cells as IRS-1 Y896 is the principal phosphorylated form of this adaptor protein under basal growth conditions. This dominance of EGFR over IGF-IR to recruit IRS-1 was further emphasised by the finding that co-treatment of Tam-R cells with EGF and IGF-II resulted in a reduced ability of IGF-II to promote Y612 phosphorylation of IRS-1 whilst there was no effect of this co-treatment regime on EGF-induced increases in IRS-1 Y896. This suggests that the EGFR/IRS-1 association in Tam-R cells prevents recruitment of IRS-1 by IGF-IR thus potentially serving to actively limit signalling via this receptor while further promoting the EGFR/MAPK pathway that is central to Tam-R cell growth. Similar findings have been reported in a prostate epithelial cell line, CPTX 1532, where EGF was shown to inhibit IGF-I-dependent degradation of IRS-1 [39]. We next examined the effect of EGFR blockade on this novel interplay between EGFR, IGF-IR and IRS-1, using the selective EGFR tyrosine kinase inhibitor gefitinib. As expected, a 7-day gefitinib treatment potently inhibited EGFR and ERK1/2 activity in Tam-R cells confirming our previous findings [30], however, an increase in AKT phosphorylation was also observed in response to this treatment regime. Interestingly, a time course study assessing the effects of gefitinib on AKT activity revealed that gefitinib was inhibitory up to day 4, corroborating previous findings in this cell line [33], but by day 7 was stimulatory. A possible explanation for this gefitinib-induced stimulation of AKT activity at this later time point was provided by the finding that gefitinib treatment also resulted in the loss of EGFR and IRS-1 association, a reduction in Y896 IRS-1, enhanced association of IRS-1 with IGF-IR and increased Y612 IRS-1 phosphorylation levels at day 7. These additional novel findings indicate that gefitinib may alter the dynamics of the EGFR/IGF-IR/IRS-1 cross-talk system, promoting IGF-IR signalling by allowing IRS-1 to re-associate with IGF-IR an event that can then serve to stimulate downstream PI3-K/AKT signalling. It should be noted that this re-establishment of IGF-IR/PI3K/AKT signalling following gefitinib treatment was not associated with any increase in phosphorylated or total IGF-IR expression. This ability of gefitinib to promote IGF-IR signalling in Tam-R cells was further evidenced by the ability of gefitinib to further enhance IGF-II-induced phosphorylation of IRS-1 Y612 and AKT when compared to IGF-II treatment alone. Activation of such a pathway may facilitate the ability of the cells to survive gefitinib challenge in the short-term and in the long-term provide a mechanism to drive resistant growth. Indeed, we have demonstrated that an “in house” tamoxifen-gefitinib resistant MCF-7 breast cancer cell line utilises the IGF-IR signalling pathway to drive resistant growth [20]. We confirmed that these effects resulted from a selective inhibition of EGFR by gefitinib as knockdown of EGFR expression using siRNA technology similarly resulted in an increase in IRS-1 Y612 phosphorylation at day 4. As siRNA knockdown of EGFR was transient and maximal at day 4, we were unable to assess whether AKT activity was enhanced by day 7 with this treatment. However, we were able to demonstrate inhibition of AKT activity with EGFR siRNA at day 4, once again consistent with our findings with gefitinib.

As gefitinib treatment appeared to rapidly enhance IGF-IR signalling in Tam-R cells we next assessed the effect of targeting IGF-IR in combination with gefitinib in an attempt to abrogate this potential resistance mechanism. Treatment of Tam-R cells with a combination of gefitinib and the selective IGF-IR tyrosine kinase inhibitor ABDP [31] prevented the activation of IGF-IR by IGF-II and blocked the gefitinib-dependent enhancement of IRS-1 Y612 and AKT phosphorylation in response to this ligand. Again, these results were shown to be a consequence of selective inhibition of EGFR and IGF-IR as similar results were observed following combined knockdown of EGFR and IGF-IR with siRNA technology. Greater inhibition of phosphorylated levels of IRS-1 Y896, EGFR Y1068 and ERK1/2 were also observed in those cells treated with the combination of ABDP and gefitinib compared with gefitinib alone, reflecting the important role played by IGF-IR in facilitating EGFR signalling activity in Tam-R cells [32]. Interestingly, ABDP and IGF-1R siRNA treatment alone inhibited both IGF-1R and EGFR signalling components, although not to the same extent as the combination treatment, confirming previous studies demonstrating that IGF-1R can modulate EGFR signalling activity in Tam-R cells [32]. The more effective inhibition of IGF-IR and EGFR signalling pathways by combination treatment also translated out into a greater inhibition of cell growth compared to either agent alone supporting the findings of Camirand et al. who reported additive or synergistic inhibitory effects on breast cancer cell growth in cells treated with a combination of the selective IGF-IR tyrosine kinase inhibitor AG1024 and gefitinib compared to either agent alone [21]. Thus, following long-term (4–5 months) combination therapy targeting EGFR and IGF-IR we have found that Tam-R cells treated with either gefitinib or ABDP alone showed evidence of re-growth, with cell numbers doubling over a 2-week time frame, confirming previous reports examining long-term gefitinib treatment in Tam-R cells [20]. However, Tam-R cells treated with a combination of gefitinib and ABDP demonstrated a sustained growth inhibition with no evidence of re-growth over the same time period. Indeed, by 19 weeks there was complete cell loss in the combination treatment arm of the study. These findings support previous studies examining combination treatment of Tam-R cells with gefitinib and AG1024 [40] and indicate that targeting the gefitinib-induced IGF-IR signalling in Tam-R cells may provide a mechanism to prevent cells surviving the initial challenge with this anti-EGFR agent and ultimately block the development of a resistant phenotype. It should be noted that the effects of IGF-1R blockade on growth will reflect an action on both IRS-1-dependent and -independent mechanisms and thus the benefit seen with combination treatment is not solely a consequence of targeting the gefitinib inductive response.

The ability of gefitinib to promote IGF-IR signalling may not be unique to the Tam-R cell line. In the LNCaP prostate cancer cell line a 7 day treatment with gefitinib reduced IRS-1 Y896 and enhanced IRS-1 Y612 phosphorylation, paralleling the findings in Tam-R cells. It has previously been reported that LNCaP cells do not express IRS-1 protein [41], however, we were able to detect very low levels of this protein in the present study possibly due to the use of more sensitive detection reagents in our Western blotting studies. It is likely that low expression levels of IRS-1 in LNCaP compared to T47D, DU145 and A549 cells would serve to greatly limit the availability of this adaptor protein and so emphasise any alterations in IRS-1 recruitment and phosphorylation patterns observed under basal conditions and following gefitinib exposure. It is likely that this novel cross-talk mechanism is not functional in T47D cells as gefitinib was ineffective at reducing Y896 IRS-1 phosphorylation levels, whilst in A549 expression levels of Y612 IRS-1 were higher than Y896 IRS-1 suggesting that EGFR was not the dominant recruiter of IRS-1 in these cells. DU145 cells do not appear to express Y612 phosphorylated IRS-1 suggesting that basal IGF-IR signalling is compromised in these cells possibly as a consequence of the serum-free conditions used in this study, which is believed to suppress IGF-IR expression and downregulate AKT activity (personal observation).

In conclusion, we have identified a novel interaction between the EGFR and the adaptor protein IRS-1 in a range of EGFR-positive breast, prostate and lung cancer cell lines. This ability of EGFR to recruit IRS-1 has also highlighted a novel cross-talk mechanism between EGFR and IGF-IR in tamoxifen-resistant MCF-7 breast, and possibly LNCaP prostate, cancer cell lines whereby recruitment of IRS-1 by EGFR may limit the availability of IRS-1 to associate with IGF-IR and as a result, potentially inhibit signalling via this receptor. This suppression of IGF-IR signalling by EGFR can be disrupted by treatment with gefitinib with the resultant association of IRS-1 with IGF-IR leading to re-establishment of IGF-IR signalling, the dominant growth regulatory mechanism of gefitinib resistance in these cells. Thus, gefitinib plays an active role in limiting its own efficacy in Tam-R cells by promoting activation of a resistance pathway. These findings clearly demonstrate that as a consequence of the high degree of cross-talk that exists between growth factor signalling pathways in cancer cells we must take into consideration that targeting a single protein in this complex signalling array may have wide-ranging and unexpected effects on a number of signal transduction pathways which may adversely influence the quality and duration of response. Deciphering both the inhibitory and inductive effects of these targeted agents provides us with the opportunity to design effective strategies to combat such resistance

mechanisms and improve response to initial therapy. Indeed, as proof of principle, in the present study we demonstrate that targeting IGF-IR signalling in combination with gefitinib, to anticipate the inductive action of gefitinib on this pathway, generates a more effective inhibition of Tam-R cell signalling activity and growth compared to gefitinib alone.

Acknowledgements The authors would like to thank Carol Dutkowski and Martin Giles for expert technical assistance. This research was generously supported by the Tenovus organisation. 'IRESSA' is a trademark of the AstraZeneca group of companies.

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