Acquired resistance to anti-EGFR mAb ICR62 in cancer cells is accompanied by an increased EGFR expression, HER-2/HER-3 signalling and sensitivity to pan HER blockers

Said A Khelwatty[1], Sharadah Essapen[1,2], Alan M Seddon[1], Zhen Fan[3] and Helmout Modjtahedi*[,1]

[1]School of Life Science, Kingston University London, London, UK; [2]St Luke's Cancer Centre, Royal Surrey County Hospital, Guildford, UK and [3]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

Background: The human epidermal growth factor receptor (EGFR) is an important target for cancer treatment. Currently, only the EGFR antibodies cetuximab and panitumumab are approved for the treatment of patients with colorectal cancer. However, a major clinical challenge is a short-term response owing to development of acquired resistance during the course of the treatment.

Methods: In this study, we investigated the molecular mechanisms underlying development of acquired resistance in DiFi colorectal cancer cells to the anti-EGFR mAb ICR62 (termed DiFi62) and to the small molecule tyrosine kinase inhibitor (TKI) gefitinib (termed DiFiG) using a range of techniques.

Results: Compared with the findings from parental DiFi and DiFiG cells, development of acquired resistance to anti-EGFR mAb ICR62 in DiFi62 cells was accompanied by an increase in cell surface EGFR and increased phosphorylation of HER-2 and HER-3. Interestingly, DiFi62 cells also acquired resistance to treatment with anti-EGFR mAbs cetuximab and ICR61, which bind to other distinct epitopes on the extracellular domain of EGFR, but these cells remained equally sensitive as the parental cells to treatment with pan-HER inhibitors such as afatinib.

Conclusions: Our results provide a novel mechanistic insight into the development of acquired resistance to EGFR antibody-based therapy in colorectal cancer cells and justify further investigations on the therapeutic benefits of pan-HER family inhibitors in the treatment of colorectal cancer patients once acquired resistance to EGFR antibody-based therapy is developed.

The epidermal growth factor receptor (EGFR/erbB1) is a transmembrane glycoprotein and belongs to the erbB family of receptors, which consists of three other members, HER-2 (erbB2/Neu), HER-3 (erbB3) and HER-4 (erbB4) (Carpenter, 1987; Haley et al, 1987; Arteaga and Engelman, 2014). Activation of the EGFR following the binding of EGFR ligand or EGFR mutation, results in the activation of several downstream signalling cascades such as RAS/RAF/MAPK, PI3K/Akt, PLCγ/PKC, Src and STAT. This ultimately contributes to the hallmarks of human cancer via tumour cell proliferation, reduced apoptosis and increased migration and invasion (Brand et al, 2011; Hanahan and Weinberg, 2011; Garouniatis et al, 2012; Khelwatty et al, 2013).

The aberrant expression and activation of the EGFR have been reported in a wide range of human cancers. This has been associated with tumour progression and poor survival in many patients, including those with colorectal cancer (Kluftinger et al, 1992; Mayer et al, 1993; Modjtahedi and Dean, 1994; Spano et al, 2005; Galizia et al, 2006; Khelwatty et al, 2014). The prominent role of EGFR in the survival and progression of human cancer has made targeting of the EGFR with mAbs or TKIs an important therapeutic approach (Sato et al, 1983; Modjtahedi et al, 1993a; Khelwatty et al, 2013; Okada et al, 2014). Currently, of the anti-EGFR mAbs, only cetuximab and panitumumab have been approved for the treatment of patients with metastatic colorectal

cancer (Wong, 2005; Wu *et al*, 2008; Modjtahedi *et al*, 2012; Arteaga and Engelman, 2014). Although the clinical efficacy of cetuximab and panitumumab has been demonstrated in many patients, the duration of response can be short, with rapid emergence of acquired resistance as a major cause of treatment failure (Cunningham *et al*, 2004; Douillard *et al*, 2010; Elez *et al*, 2010; Gravalos *et al*, 2010; Osumi *et al*, 2013). It is therefore essential not only to unravel the possible mechanisms of acquired resistance to therapy with anti-EGFR mAbs in colorectal cancer but also to develop novel and more effective therapeutic strategies for such patients (Lu *et al*, 2007; Yonesaka *et al*, 2011; Montagut *et al*, 2012; Khelwatty *et al*, 2013).

In the early 1990s, we developed a large panel of anti-EGFR antibodies against the extracellular domain of the EGFR for use in cancer therapy (Modjtahedi *et al*, 1993a, b, 2003; Modjtahedi and Dean, 1994). Of these, antibody ICR62 was studied extensively due to its superior anti-tumour activity against the EGFR over-expressing tumour cells both *in vitro* and *in vivo* and clinical trials have also been conducted with mAb ICR62, and one of the humanised version of this antibody imgatuzumab (GA201) (Modjtahedi *et al*, 1993b, 1996; Modjtahedi and Dean, 1994; Paz-Ares *et al*, 2011; Delord *et al*, 2014). Previously, we reported that the EGFR-overexpressing DiFi colorectal cancer cells (wild type for KRAS, BRAF and PI3K) are dependent upon EGFR-mediated cell signalling for proliferation and survival and that blockade of the EGFR by anti-EGFR mAb ICR62 or cetuximab, or inhibition of EGFR with EGFR TKIs leads to cell death via apoptosis (Wu *et al*, 1995; Liu and Fan, 2001; Liu *et al*, 2001; Cunningham *et al*, 2006; Khelwatty *et al*, 2011). In addition, we and others have shown DiFi colorectal cancer cells to be highly sensitive to treatment with a panel of our in-house anti-EGFR mAbs including ICR61, which bind to another epitope on the extracellular domain of the EGFR distinct from the ICR62 binding site (Fan *et al*, 1993; Modjtahedi *et al*, 1993b). In this study, we investigated the mechanisms of acquired resistance to therapy with anti-EGFR mAb ICR62 by treating DiFi cells with chronic doses of ICR62 or the EGFR TKI gefitinib. We characterised molecular profiling of parental DiFi cells and their ICR62 and gefitinib-resistant sublines. We show that tumour cells that acquire resistance to anti-EGFR mAb ICR62 also acquire resistance to treatment with mAbs cetuximab and ICR61, both of which bind to other distinct epitopes on the extracellular domain of the EGFR, but interestingly, these cells remain sensitive to pan-HER family inhibitors. In addition, we found that acquired resistance is accompanied by an increased level of cell surface EGFR and increased phosphorylation of HER-2 and HER-3.

MATERIALS AND METHODS

Antibodies and other reagents. The rat mAb ICR10 (IgG2a) was raised against the external domain of the EGFR on the head and neck carcinoma cell line HN5, and mAbs ICR62 (IgG2b) and ICR61 (IgG2b) were raised against two other distinct epitopes on the external domains of the human EGFR on the breast carcinoma cell line (MDA-MB468) as described previously (Modjtahedi *et al*, 1993b). The anti-EGFR mAb cetuximab was purchased from Merck Serono (London, UK). The irreversible pan-HER inhibitor afatinib (BIBW2992) was kindly provided by Boehringer Ingleheim (Vienna, Austria). The reversible anti-EGFR TKI, gefitinib (Iressa/ZD1839) was purchased from R&D Systems (Oxford, UK). A panel of small molecule TKIs, neratinib, sapitinib, imatinib, dasatinib, canertinib and lapatinib, was purchased from Stratech Scientific Ltd (Newmarket, UK) and crizotinib and PF04217903 mesylate was purchased from R&D systems. Mouse antibodies against phospho-Tyr-100 and β-actin and rabbit antibodies against total Akt, MAPK, phospho-MAPK (Thr202/Tyr204), phospho-Akt

(Ser473), anti-phospho EGFR antibodies, anti-phospho HER-2 and HER-3 antibodies were obtained from New England Biolabs (Hitchin, UK). Mouse anti-EGFR (F4) antibody was purchased from Sigma-Aldrich (Gillingham, UK).

Tumour cell lines and establishment of variants resistant to EGFR inhibitors. The human colorectal tumour cell line, DiFi, was established from a patient with familial adenomatous polyposis (FAP) as described previously (Fan *et al*, 1993). All the cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and the antibiotics, penicillin, streptomycin and neomycin (Sigma-Aldrich) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. DiFi variant sublines were established following chronic treatment of DiFi cells with increasing concentrations of mAb ICR62 (DiFi62) or the EGFR TKI gefitinib (DiFiG) over a period of 6–10 months. In parallel, the DiFi parental cells were kept in culture until the resistant variants were established. The identity of DiFi parental cells was authenticated using Short Tandem Repeat (STR) profiling less than 6 months ago (LGC, London, UK).

Growth response studies. The effect of other anti-EGFR mAbs and small molecule TKIs on the growth of human colorectal tumour-resistant variant cell lines *vs* the parental cell line was investigated using sulphorodhamine B (SRB; Sigma Aldrich) colorimetric assay as described previously (Khelwatty *et al*, 2011). Briefly, tumour cells were seeded at a density of 5×10^3 cells per well in 100 μl growth medium supplemented with 10% FBS in a 96-well plate. Cells were incubated at 37 °C until the cells in the wells containing only control medium were near confluent. The cells were then fixed with 10% trichloroacetic acid (TCA) and stained with 0.04% SRB in 1% acetic acid and solubilised with 10 mM Tris-base and the absorbance of each well was measured at 565 nm using an Epoch plate reader (Thermo Fisher, Loughborough, UK). Data were analysed using Gen5 (Biotek, Swindon, UK) and Calcusyn software (Biosoft, Cambridge, UK).

Flow cytometry. Expression level of the HER family members was determined by flow cytometry as described previously (Khelwatty *et al*, 2011). Briefly, 1×10^6 tumour cells in 1 ml of PBS were incubated with primary antibodies or control medium for 1 h by rotation at 4 °C, followed by incubation with FITC-conjugated IgG secondary antibody for 1 h at 4 °C. A minimum of 10 000 events were recorded by excitation with an argon laser at 488 nm, and analysed using the FL-1 detector (FITC detector; 525 nm) of BD FACScalibur flow cytometer (Becton-Dickinson, Oxford, UK) using CellQuest Pro software.

Immunofluorescent staining of EGFR. Tumour cells were grown to near confluence in DMEM/10% FBS in a Lab-Tek Parmanox eight-well chamber culture slide (VWR, East Grinstead, UK). The cells were incubated with anti-EGFR antibodies for 1 h on ice or 37 °C (for internalisation studies) and then fixed with 4% paraformaldehyde for 10 min at room temperature. Cell membranes were permeabilised with 0.5% Triton X-100 (Sigma-Aldrich) in PBS/1% BSA for 20 min at room temperature and blocked with PBS/3% BSA for 30 min. The tumour cells were then incubated with FITC conjugated secondary antibody (AbD Serotec, Oxford, UK) for 1 h at 4 °C. Following washes in PBS, cells were incubated with 1 μg ml^{-1} of Hoescht 33258 nuclear stain (Sigma-Aldrich) for 5 min at room temperature and mounted with H-1000 mounting medium (Vector Laboratories, Peterborough, UK), cover slipped (VWR) and photographed using FITC filter array on a Nikon eclipse i80 fluorescent microscope.

Western blotting. Tumour cells were grown to near confluence in six-well culture plates (Fisher Scientific, Loughborough, UK) containing 5 ml of 10% FBS/DMEM growth medium. The cells

were lysed using lysis buffer (Fisher Scientific) containing a protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were heated in SDS sample buffer for 10 min at 72 °C. Protein samples were separated on 4–12% Bis-Tris gels (Fisher Scientific) using the XCell II Surelock Mini-Cell system (Fisher Scientific) and transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were probed sequentially as described previously (Cunningham et al, 2006). The specific signals were detected using G:BOX-CHEMI-XT4 gel documentation system (Syngene, Cambridge, UK).

Receptor tyrosine kinase proteome array. To determine the phosphorylation status of human receptor tyrosine kinases (RTKs), we used the Proteome Profiler 96 purchased from R&D systems. Cells grown in culture flasks (25 cm^2) under normal conditions to near confluence were lysed at a density of 1×10^7 cells ml^{-1}. The proteome array assay was carried out according to the manufacturers' manual. The data were analysed using G:BOX-CHEMI-XT4 and ImageJ software.

DNA sequencing analysis. For DNA sequencing, DNA extraction from DiFi parental and drug-resistant variants was performed using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Manchester, UK) as described in the manufacturer's manual. Sequencing libraries were prepared with 10 ng of each sample using ready-to-use primers from Ion Ampliseq Cancer Hotspot Panel v2 and Ion Ampliseq Library Kit following manufacturer's instructions (Life Technologies, Paisley, UK). Following library preparation, Ion OneTouch system was used to prepare a template and sequencing was performed using an Ion 316 chip on an Ion PGM sequencer and results were analysed using Ion Ampliseq CHPv2 regions and hotspots, hg19 as reference in single sample analysis. In addition, analysis was also carried out by comparing DiFi parental (normal) and DiFi drug-resistant variants (tumour) in paired analysis using Ion Reporter software (Life Technologies).

RESULTS

Resistance to anti-EGFR mAb ICR62 is accompanied with up-regulation of EGFR. Both ICR62 and gefitinib inhibited the growth of parental DiFi cells with IC$_{50}$ values of 1 nM and 25 nM, respectively (Table 1). To elucidate the molecular mechanisms underlying the acquired resistance to anti-EGFR therapy, we chronically exposed parental DiFi cells to increasing concentrations of anti-EGFR mAb ICR62 or the reversible anti-EGFR TKI

gefitinib for a period of 6–10 months. As shown in Figure 1A and B, both DiFi62 and DiFiG resistant variants acquired resistance to treatment with ICR62 and gefitinib, with increased IC$_{50}$ values being >209.96 nM for ICR62 and 113.74 nM for gefitinib, respectively (Table 1).

Following the establishment of the DiFi-resistant variants, we performed comparative analysis measuring the cell surface expression of all known members of the erbB family in the DiFi parental cells and resistant variants DiFi62 and DiFiG. Interestingly, while the cell surface expression of the HER-2, HER-3 and HER-4 remained mostly unchanged, there was a marked increase in the expression of EGFR in DiFi62 variant cells (Figure 1C). To determine whether the increase in the cell surface expression of the EGFR was associated with the total EGFR levels, we determined the total EGFR levels in the DiFi parental and drug-resistant variants, DiFi62 and DiFiG. Interestingly, western blot analysis showed that while total EGFR protein was detected abundantly in the DiFi parental cells and DiFiG resistant variant, the level of total EGFR in DiFi62 cells was undetectable using the mouse anti-EGFR (F4) antibody, which is directed against the intracellular domain of the EGFR (residues 985 to 996) (Figure 1D). In addition, the result of immunofluorescence staining with mAb F4 indicated that although it can bind to the EGFR on DiFi parental cells, it was unable to detect the EGFR in DiFi62 variant cells (Figure 1D). However, subsequent re-probing of the same membrane using the rat anti-EGFR (ICR10) antibody, which is directed against the extracellular domain of the EGFR, revealed a marked increase in the level of EGFR in DiFi62 cells compared to the level of EGFR in DiFi parental cells and in DiFiG resistant variant, consistent with the findings of the flow cytometry (Figure 1C and D). Interestingly, the band detected by anti-EGFR mAb ICR10 was found to be a 'fast-migrating' form of the EGFR (Figure 1D). These finding suggested that chronic treatment of DiFi cells with anti-EGFR mAb ICR62 was indeed accompanied by the appearance of a modified EGFR.

Up-regulation of EGFR in DiFi62 cells is owing to impaired endocytosis of the EGFR. Considering the lack of detectable total EGFR protein by western blotting using the mouse anti-EGFR (F4) antibody, which is directed against the intracellular domain of the EGFR (Figure 1D), we wondered whether the phosphorylation status of the EGFR at several sites has altered in DiFi62 cells. Strikingly, among the listed EGFR phosphorylation sites, phosphotyrosine was either undetectable or markedly diminished in DiFi62 cells compared with the findings in parental DiFi or DiFiG

Table 1. Sensitivity of DiFi parental colorectal cancer cells compared with DiFi62 and DiFiG drug-resistant variants to a panel of mAbs and TKIs as IC$_{50}$ values (nM)

Inhibitors	IC$_{50}$(nM)		
	DiFi	DiFi62	DiFiG
mAbs			
ICR61 (anti-EGFR mAb)	2.19	>209.96	>209.96
ICR62 (anti-EGFR mAb)	1.09	>209.96	3.12
Cetuximab (anti-EGFR mAb)	<1.00	>209.96	1.16
Small molecule TKIs			
Afatinib (irreversible pan-HER inhibitor)	10.00	8.00	61.00
Canertinib (irreversible pan-HER inhibitor)	4.66	<1.00	89.65
Neratinib (irreversible pan-HER inhibitor)	8.35	1.82	50.65
Sapitinib (equipotent reversible pan-HER inhibitor)	1.44	<1.00	8.29
Lapatinib (reversible dual EGFR and HER-2 inhibitor)	41.55	46.07	374.29
Gefitinib (reversible EGFR inhibitor)	24.98	17.16	113.74
Imatinib (BCR-Abl inhibitor)	>1049.80	>1049.80	>1049.80
Dasatinib (dual Bcr-Abl and Src inhibitor)	>1049.80	>1049.80	>1049.80
PF04217903 mesylate (Alk inhibitor)	>1049.80	>1049.80	>1049.80
Crizotinib (dual cMet and Alk inhibitor)	587.54	488.11	916.76

Abbreviations: EGFR = epidermal growth factor receptor; mAb = monoclonal antibody; TKI = tyrosine kinase inhibitor.
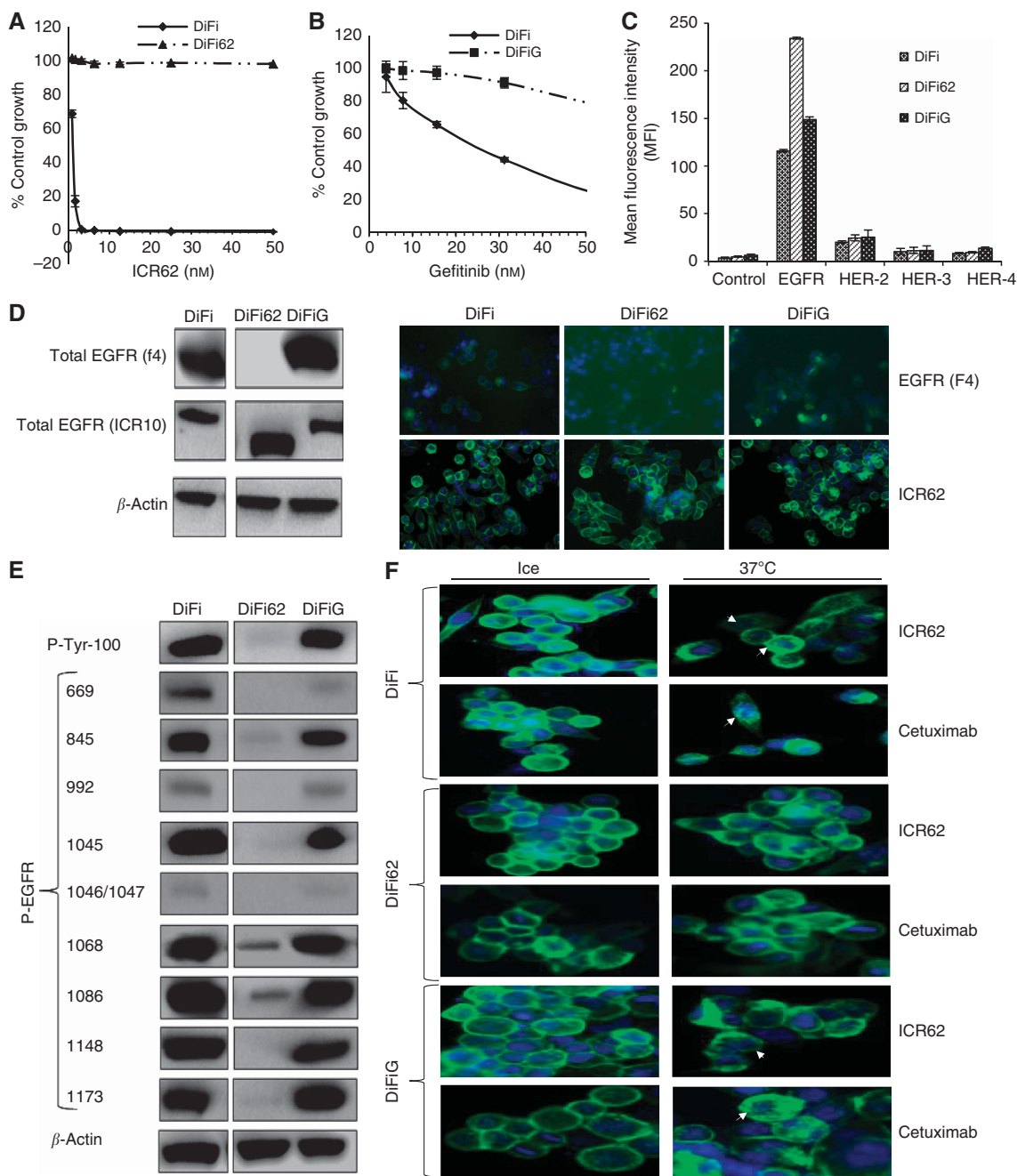
Figure 1. Growth response of parental cells and drug-resistant variants to doubling dilutions of anti-EGFR mAb ICR62 and gefitinib. Growth response of DiFi parental cells and the drug-resistant variants DiFi62 and DiFiG cells to doubling dilutions of anti-EGFR mAb ICR62 (**A**) and gefitinib (**B**).The cell surface expression of erbB family members measured by flow cytometry analysis in DiFi parental and its drug-resistant variants DiFi62 and DiFiG cells (**C**). The expression of total EGFR in DiFi parental and its drug-resistant variants DiFi62 and DiFiG cells using a mouse anti-EGFR antibody (F4) and rat anti-EGFR antibody ICR10 and ICR62 by western blotting and immunofluorescence (**D**), and basal levels of phosphotyrosine and phosphorylated EGFR sites (**E**) by western blotting. Immunofluorescence staining of the EGFR in DiFi parental and its drug-resistant variants DiFi62 and DiFiG cells following incubation with anti-EGFR mAbs ICR62 ($200 \, nmol \, l^{-1}$), cetuximab ($200 \, nmol \, l^{-1}$) for 1 h on ice or $37 \, ^{\circ}C$ (**F**). Internalised EGFR is indicated by the intracellular endocytic vesicles staining for EGFR as shown by the white arrows.

cells (Figure 1E). For example, the phosphorylation of tyrosine residues 1045 (a Cbl-binding site) was undetectable and the phosphorylation of tyrosine residues 1068 (a growth factor receptor binding protein-2-binding site) was significantly reduced in DiFi62 drug-resistant variant cells, respectively. In contrast, DiFiG cells retained similar levels of EGFR phosphorylation found in parental DiFi cells, except the level on Thr-669 residue (ligand-induced regulation of receptor internalisation; Figure 1E). Using immunofluorescence staining, we further found that EGFR remained in the extracellular membrane upon treatment with ICR62 or cetuximab in the DiFi62 cells compared with reduced immunofluorescence staining of EGFR in parental DiFi and DiFiG cells after the same treatment (Figure 1F).

Taken together, these results indicate that the intracellular cytoplasmic domain of the EGFR in DiFi62 cells is altered and the increased level of EGFR in DiFi62 cell surface could be a consequence of an impaired internalisation and subsequent degradation of EGFR in DiF62 cells.

Partial loss of EGFR gene is associated with resistance to anti-EGFR mAb ICR62. On the basis of the above findings, we sought to further investigate whether there were any genetic alterations that had occurred as a consequence of chronic treatment with anti-EGFR mAb ICR62 or small molecule TKI gefitinib in DiFi cells. For this purpose, we performed mutational analysis by using Ion Ampliseq cancer panel targeting 50 cancer hotspot genes, such as KRAS, NRAS, BRAF, PIK3CA, EGFR, ErbB2, ErbB3, ErbB4 and TP53. DNA sequencing revealed a missense mutation of C > G substitution in chromosome 17 at nucleotide 97 of TP53 gene causing a substitution of proline to alanine at amino acid 97 in both DiFi62 and DiFiG drug-resistant variant cells (Table 2). In addition, a synonymous mutation of A > G substitution in chromosome 4 at nucleotide 858 of F-box and WD repeat domain containing 7 (FBXW7) gene and a non-coding mutation in chromosome 4 in the intronic region of KDR gene was found in DiFiG and DiFi62 drug-resistant variants respectively (Table 2). Interestingly, in DiFi62 drug-resistant variant cells, a novel loss of copy number of 48.584 kb in length in the EGFR and EGFR-AS1 genes corresponding to the regions encoding for the intracellular domain of the EGFR protein was also detected, which was not present in DiFi parental or DiFiG drug-resistant variant cells (Table 2).

These findings further confirmed that the intracellular domain of the EGFR is indeed altered causing diminished receptor internalisation and/or degradation and as a result DiFi62 drug-resistant variant cells have an increased extracellular expression of EGFR.

Resistance to anti-EGFR mAb ICR62 is accompanied by upregulation of pHER-2 and pHER-3. Having shown that acquired resistance to anti-EGFR mAb ICR62 in DiFi cells is accompanied by increased level of cell surface EGFR, but not that of HER-2 or HER-3, we next examined whether the acquired resistance to ICR62 was associated with increased activation of HER-2, HER-3 and/or other alternative receptor tyrosine kinases that activate overlapping signal transduction pathways downstream of EGFR. We performed a high-throughput comparative analysis using a phosphor-RTK array kit measuring a panel of phosphorylated RTKs in parental DiFi cells vs the resistant sublines (Figure 2A and B). Of the phosphorylated RTKs measured, the erbB family members were found to be phosphorylated in DiFi parental cells and in DiFi62 and DiFiG cells (Figure 2A). As shown in Figure 2A–C, resistance to ICR62 was accompanied by a reduction in the level of pEGFR but increased phosphorylation of both HER-2 and HER-3 in DiFi62 cells (Figure 2A and B). In contrast, the phosphorylation of EGFR and HER-2 in DiFiG cells remained the same while the phosphorylation of HER-3 appeared to be lower compared with the findings in DiFi parental cells (Figure 2A and B). As shown in Figure 2C, phosphorylation of other RTKs in DiFi parental or its drug-resistant sublines was not detectable using the RTK array kit. Taken together, these data indicate that acquired resistance to ICR62 was accompanied by an increased level of cell surface EGFR and increased phosphorylation

of both HER-2 and HER-3. We further validated the findings of the RTK array kit by western blot analysis to measure the levels of phosphorylated HER-2, and HER-3, as well as that of MAPK and Akt, two major molecules mediating cell signal transduction downstream of EGFR. The results of western blotting corroborate with the findings from the phospho-RTK array (Figure 2C). The increased phosphorylation of HER-2 and HER-3 in DiFi62 cells relative to DiFi parental cells was accompanied by increased phosphorylation of MAPK and Akt (Figure 2C). We also examined the phosphorylation of several other downstream signal transduction pathways such as JAK/STAT, MET and Src family kinases. Although no striking differences were noted in the activation of the STATs (data not shown), there was an increased phosphorylation of Src (Ser 17) but not MET phosphorylation in DiFi62 and DiFiG cells compared with parental DiFi cells (Figure 2D).

ICR62-resistant DiFi cells acquire resistance to other anti-EGFR mAbs but remain sensitive to small molecule HER inhibitors. As acquired resistance to treatment with anti-EGFR mAbs would lead to a short-term response, we next investigated response of DiFi62 cells to treatment with other anti-EGFR mAbs including mAb ICR61 and cetuximab (Modjtahedi et al, 1993b). It would be interesting to determine whether some of these anti-EGFR mAbs in our panel, which are directed against epitopes distinct from the ICR62 binding site, would be effective in inhibiting the growth of DiFi62 cells. As shown in Figure 3A, all three mAbs bind to the extracellular domain of the EGFR in both parental DiFi cells and its two sublines. As expected, parental DiFi cells were highly sensitive to treatment with all anti-EGFR mAbs used in this study (Figure 3B, Table 1). However, DiFi62 cells were found to acquire resistance to treatment with either ICR61 or cetuximab (Figure 3C). In contrast, DiFiG cells were sensitive to treatment with either cetuximab or ICR62 but not as sensitive to ICR61 (Figure 3D, Table 1). Taken together, these results suggest that acquired resistance in DiFi62 cells is not owing to an inability of ICR62 and other antibodies to bind to the extracellular domain of the EGFR in these cells.

ICR62-resistant DiFi cells remain sensitive to small molecule HER inhibitors by inhibiting the HER-2/HER-3 phosphorylation. Having demonstrated that DiFi62 variant cells also acquire resistance to treatment with other anti-EGFR antibodies and the resistance was accompanied by increase in the levels of pHER-2 and pHER-3, we next examined the sensitivity of DiFi62 and DiFiG cells to treatment with a panel of small molecule TKIs, including the irreversible pan-HER inhibitors afatinib, canertinib, and neratinib, the equipotent reversible pan-HER inhibitor sapitinib, the reversible dual EGFR and HER-2 inhibitor lapatinib, and several other TKIs including the Bcr-Abl inhibitor imatinib and dual Bcr-Abl and Src inhibitor dasatinib, the Alk inhibitor PF04217903 mesylate, and the dual cMet and Alk inhibitor crizotinib. Of the HER-inhibitors, DiFi parental cells were highly sensitive to the pan-HER inhibitors, in particular sapitinib and afatinib (Figure 4A, Table 1). More importantly, drug-resistant

Table 2. Mutational analysis of DiFi62 and DiFiG drug-resistant variants normalised against DiFi parental cells

Sample	Gene	Genotype	Amino Acid	Comment	P-value
DiFiG	FBXW7	c.858A > G	—	An SNV causing a synonymous mutation at transcript NM_033632.3	0.00001
	TP53	c.97C > G	Pro33Ala	An SNV causing a missense mutation at transcript NM_001276760.1	0.00001
DiFi62	KDR	—	—	Non-coding mutation (NM_002253.2)	0.00001
	TP53	c.97C > G	Pro33Ala	An SNV causing a missense mutation at transcript NM_001276760.1	0.00001
	EGFR, EGFR-AS1	7p11.2(55211044-55259628)x1	—	48.584 kb deletion	CNV confidence = 12.67

Abbreviations: CNV = copy number variation; EGFR = epidermal growth factor receptor; SNV = single-nucleotide variant.
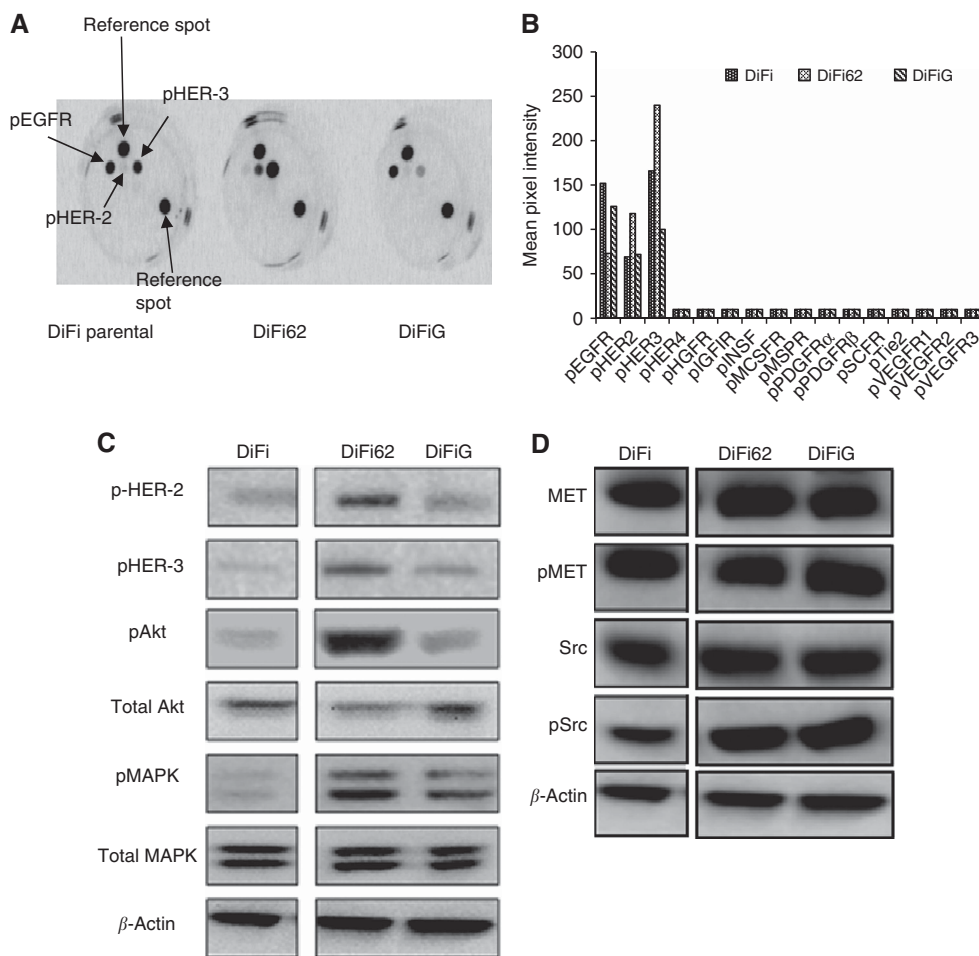
Figure 2. The phosphorylation status of a panel of RTKs in DiFi parental and the drug-resistant variants DiFi62 and DiFiG. The phosphorylation status of a panel of RTKs in DiFi parental and the drug-resistant variants DiFi62 and DiFiG cells measured by human phospho-RTK array (**A**) and presented based on mean pixel intensities (**B**). The expression of phosphorylated HER-2, HER-3, total and phosphorylated Akt and MAPK (**C**) and total and phosphorylated SRC and MET and β-actin (**D**) in DiFi parental and its drug-resistant variants DiFi62 and DiFiG cells. Tumour cells grown in 10% FBS/DMEM were lysed and 20 μg of each protein lysate was analysed by western blotting as described in 'Materials and Methods'.

variant DiFi62 cells were equally sensitive to the pan-HER inhibitors but not the reversible dual EGFR and HER-2 inhibitor lapatinib (Figure 4B, Table 1). Although DiFiG cells showed a marked decreased sensitivity to the other TKIs, they were found to be relatively sensitive to treatment with the pan-HER inhibitors neratinib, canertinib, sapitinib and afatinib (Figure 4C, Table 1).

We next examined the effects of these drugs on inhibiting phosphorylation of EGFR and related proteins. We found that compared with parental DiFi cells, treatment of DiFi62 cells with ICR62 or cetuximab could not inhibit phosphorylation of EGFR (represented by EGFR-Y1068), HER-2, HER-3 or MET, nor phosphorylation of subsequent Akt and MAPK-mediated downstream signalling pathways (Figure 4D). In contrast, the irreversible pan-HER inhibitor afatinib was effective in inhibiting the activity of EGFR, HER-2, HER-3, MET, Akt and MAPK in DiFi62 cells. We found that the reversible EGFR TKI gefitinib was equally effectively in inhibiting phosphorylation of HER-2, HER-3 and MET in parental DiFi, DiFi62 and DiFiG cells (Figure 4D). Although the treatment with anti-EGFR mAbs ICR62 and cetuximab or small molecule TKI gefitinib was able to modestly reduce the activity of MAPK and MET, treatment with the irreversible pan-HER inhibitor afatinib completely inhibited the activity of MAPK but not MET in DiFiG cells (Figure 4D). No difference was observed in the activity of Src following treatment with the anti-EGFR mAbs or small molecule TKIs in parental DiFi cells and in DiFi62 or DiFiG cells (Figure 4D). These results

suggest that HER-2 and HER-3 activation has an important role in the manifestation of drug resistance to anti-EGFR mAb therapy in DiFi62 cells.

DISCUSSION

As noted earlier, the human EGFR is an important therapeutic target in a wide range of human cancers, and of the EGFR inhibitors only the anti-EGFR mAbs cetuximab and panitumumab have currently been approved for the treatment of patients with metastatic colorectal cancer. However, development of drug resistance to these agents, which is very common in many such patients, is a major cause of treatment failure following a short course of treatment (Modjtahedi and Essapen, 2009; Misale *et al*, 2014). Although the role of EGFR and other HER family members in regulating progression of many types of solid tumours, including colorectal cancer, is well established, their roles in the development of acquired drug resistance have not been extensively studied (Khelwatty *et al*, 2013; Arteaga and Engelman, 2014). A few earlier studies have reported that acquired drug resistance to anti-EGFR therapy in colorectal cancer, head and neck squamous cell carcinoma and non-small cell lung cancer models can be attributed to dysregulation of EGFR endocytosis, upregulation of EGFR and transactivation of HER-2 and/or HER-3 (Lu *et al*, 2007; Wheeler *et al*, 2008; Yonesaka *et al*, 2011) and
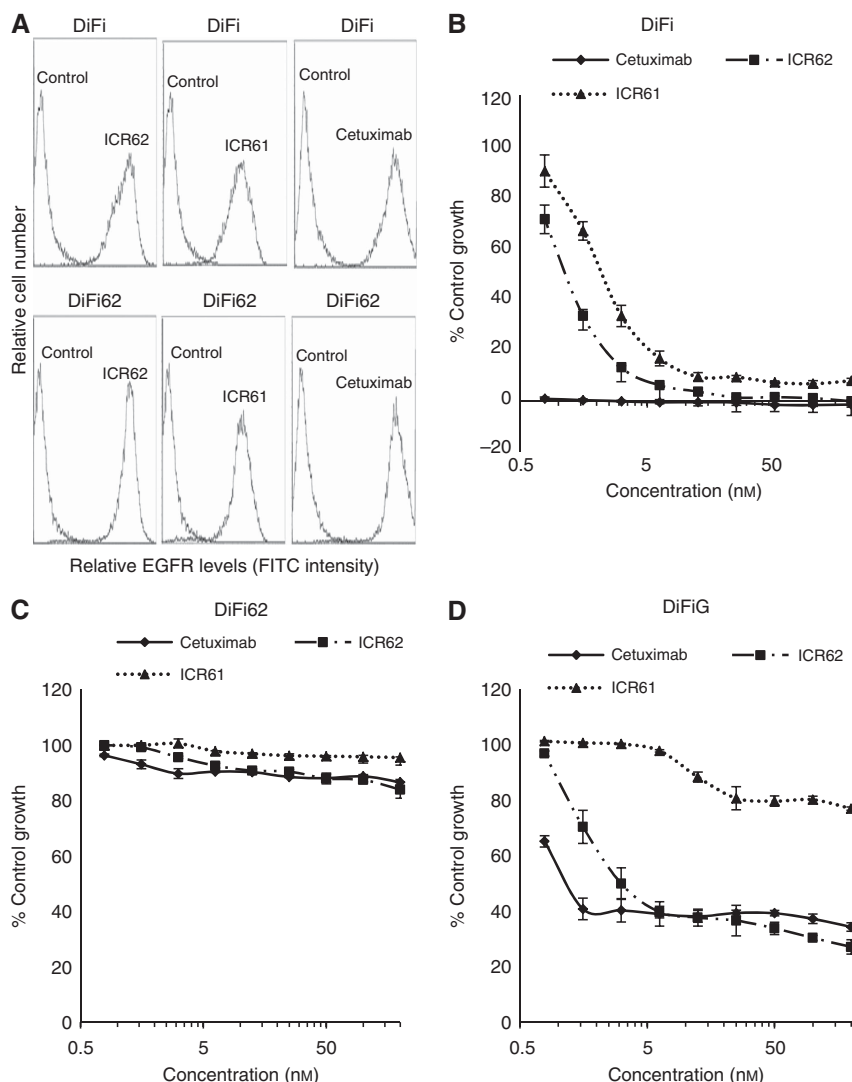
Figure 3. The detection of EGFR by anti-EGFR mAbs ICR62 and cetuximab measured by flow cytometry analysis. The detection of EGFR by anti-EGFR mAbs ICR62 and cetuximab measured by flow cytometry analysis in DiFi parental and the drug-resistant variants DiFi62 and DiFiG cells (**A**). Growth response of DiFi parental (**B**) and the drug-resistant variants DiFi62 (**C**) and DiFiG (**D**) cells to doubling dilutions of anti-EGFR mAbs ICR61, ICR62 and cetuximab.

mutations on the extracellular domain of the EGFR or the intracellular tyrosine kinase domain of the EGFR (Montagut *et al*, 2012; Arena *et al*, 2015).

In our current study, we developed resistant sublines and investigated the mechanisms of acquired resistance to treatment with the anti-EGFR mAb ICR62 and the EGFR TKI gefitinib in DiFi colorectal cancer cells. We found an increased level of cell surface EGFR in DiFi cells following the treatment with chronic doses of the anti-EGFR mAb ICR62 (Figure 1C). One reason for such an alteration in the expression level of the EGFR could be owing to dysregulated endocytosis and degradation (Wheeler *et al*, 2008). Earlier studies indicated that EGFR endocytosis and degradation is regulated by the process of ubiquitination where phosphorylated Cbl proteins bind to EGFR either directly at phosphorylated residue 1045 or indirectly to phosphorylated residue 1068 via adaptor protein Grb2 (Levkowitz *et al*, 1999; Waterman *et al*, 2002). Although alterations in the kinase domain of EGFR are usually attributed to acquired drug resistance to TKI therapy, in this study, for the first time to our knowledge, we provide evidence that alteration in the intracellular domain of the EGFR may be also associated with development of resistance to EGFR antibody-based therapy. We found that in DiFi62 cells, the phosphorylation sites of the EGFR, in particular Y1045 and Y1068, were predominantly

undetectable or markedly diminished compared with that in parental DiFi cells (Figure 1E). This observation strongly suggests that increased level of cell surface EGFR in DiFi62 cells may be caused, in part, by dysregulated receptor internalisation and/or degradation (Figure 1F) as a result of alterations in the tyrosine kinase domain of the EGFR. In addition, the data from DNA sequencing of the DiFi62 drug-resistant variant cells revealed a gene copy number variation lacking 48.584 kb in chromosome *7p11.2* 55211044-55259628, which corresponds to the region encoding for the intracellular domain of the EGFR protein (Ekstrand *et al*, 1992; Kumar *et al*, 2008; Cho *et al,* 2011; Table 2). Although many studies have investigated and reported the role of gene copy number variations as a predictive biomarker for response to anti-EGFR therapies in cancer, to our knowledge, its relationship with acquired drug-resistance in colorectal cancer has not been previously reported (Algars *et al*, 2011; Shen *et al,* 2014). Moreover, this finding could provide further explanation for the lack of detection of 'fast-migrating' EGFR band by mAb F4, which is detected by mAb ICR10, and lack of detection of several EGFR autophosphorylation sites in this study (Figure 1D–E).

In a recent study, Montagut *et al* found that acquired resistance of DiFi cells to cetuximab is owing to emergence of a mutation in
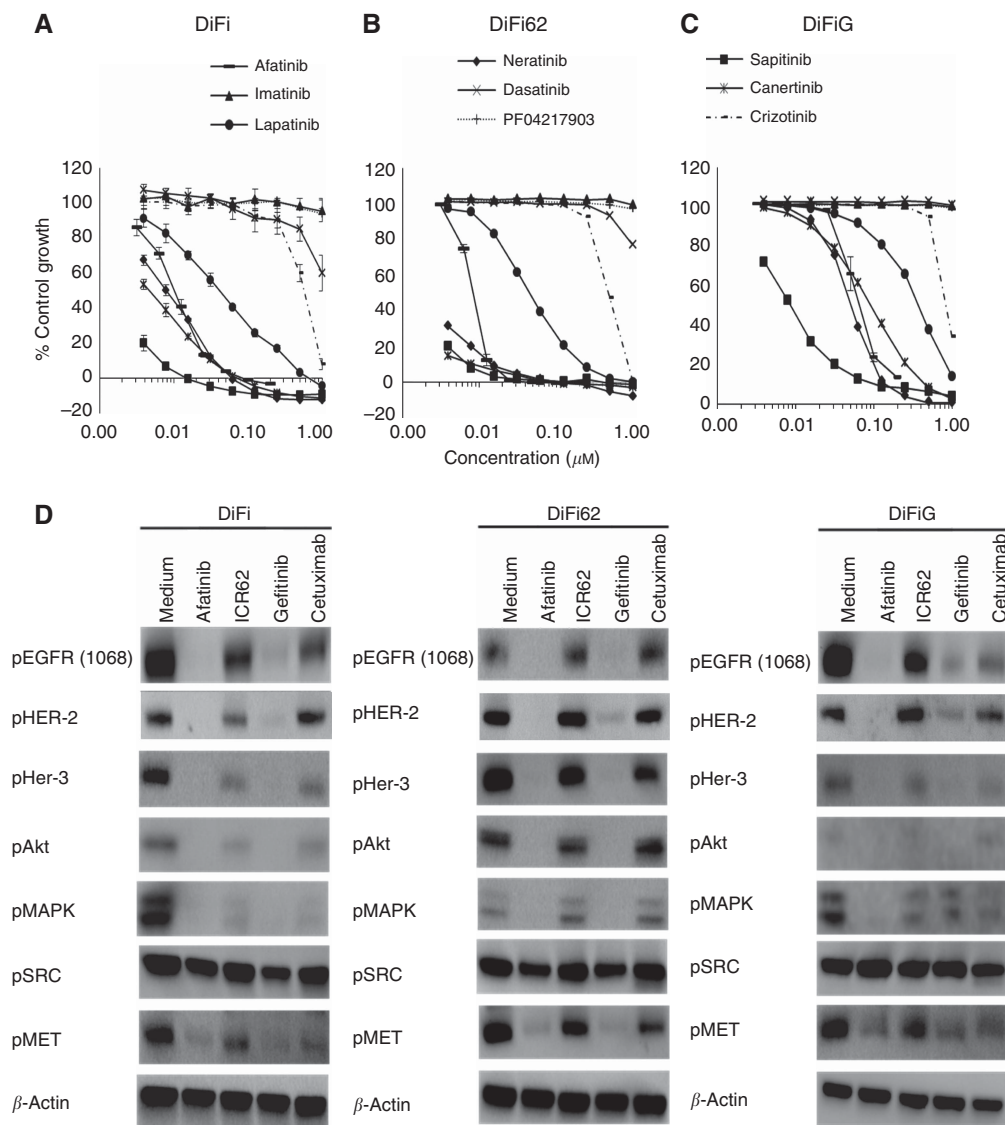
Figure 4. Growth response of parental and drug-resistant variants to doubling dilutions of small molecule TKIs. Growth response of DiFi parental (**A**) and the drug-resistant variants DiFi62 (**B**) and DiFiG (**C**) cells to doubling dilutions of a panel of small molecule TKIs. The phosphorylation status of EGFR, HER-3, Akt, MAPK, Src and MET in DiFi parental and its drug-resistant variants DiFi62 and DiFiG cells treated with anti-EGFR mAbs or TKIs (**D**).

the EGFR extracellular domain (S492R) that prevents the binding of cetuximab to the EGFR. Interestingly, they also reported that cells with such mutation retained binding to and were growth inhibited by panitumumab (Montagut *et al*, 2012). More recently, Arena *et al* have reported the emergence of a complex pattern of mutations in EGFR, KRAS, NRAS, BRAF and PIK3CA genes in cetuximab-resistant colorectal cancer including multiple mutations in the EGFR extracellular domain (K467T, R451C, G465R, S464L and I491M). Of these, with the exception of R451C mutated cells, cells containing other mutations were incapable of binding with cetuximab, while panitumumab was able to bind to only a subset of EGFR mutant cells (K467T and R451C) (Arena *et al*, 2015). In this study, we show that both ICR62 and cetuximab bind equally well to both parental DiFi cells and their ICR62 variants (DiFi62; Figure 3A). Indeed, when treated with cetuximab, which binds to different epitopes on the EGFR extracellular domain, DiFi62 cells maintained EGFR, HER2 and HER-3 activities, as well as Akt- and MAPK-mediated signalling (Figure 4D). In contrast, when treated with small molecule TKIs, such as irreversible pan-HER inhibitor afatinib, DiFi62 exhibited not only reduced phosphorylation of EGFR, HER-2 and HER-3, but also reduced Akt- and MAPK-mediated cell signalling (Figure 4D). These findings also provide an

explanation to the higher sensitivity of DiFi62 cells to pan-HER inhibitors compared with mono or dual inhibitors of EGFR or EGFR/HER2, such as lapatinib, used in this study (Figures 3B and 4B). In addition, as inhibition of HER-2 or downstream pathways may lead to transcriptional and posttranslational upregulation of HER-3, dual inhibition of EGFR and HER-2 with lapatinib does not eliminate the compensatory upregulation of HER-3 (Garrett *et al*, 2013). Therefore, our results suggest that increased activation of HER-3 signalling could be a major contributing factor by which colorectal cancer could acquire resistance to treatment with anti-EGFR antibody ICR62 or cetuximab. As evidence supporting this speculation, we found DiFi62 and DiFiG cells remain sensitive to treatment with small molecules and in particular pan-HER inhibitors (Figure 4A and B).

Previous studies in other models have shown that increased levels of EGFR could lead to transactivation of HER-2 and HER-3 (Wheeler *et al*, 2008; Brand *et al*, 2013). In this study, we have found that acquired resistance to the anti-EGFR antibody ICR62 in colorectal cancer cells was accompanied by an increased level of cell surface EGFR and increased activation of pHER-2 and pHER-3, which was accompanied by increased phosphorylation of MAPK and Akt in DiFi62 cells. Our result would therefore support

that HER-2/HER-3 phosphorylation has a major role in driving the survival and proliferation of DiFi62 cells (Yonesaka *et al*, 2011). Other reports also suggest that HER-3 has an important role in the regulation of response to anti-EGFR therapies. For example, in a recent study, cetuximab-resistant head and neck cancer cells were found to escape treatment by anti-EGFR therapy through HER-3 activation (Kjaer *et al*, 2013). Similarly, in another study, heregulin-EGFR-HER-3 autocrine signalling axis was found to mediate acquired resistance to lapatinib in HER-2-positive breast cancer models (Xia *et al*, 2013). More recently, HER-3 phosphorylation in colorectal cancer cells was shown to be strictly dependent on association with HER-2, and that HER-2/HER-3 signalling reversed the effect of EGFR blockade on inhibiting colorectal cancer cell growth (Zhang *et al*, 2014). Taken together, these studies suggest that HER-3 mediates tumour response to anti-EGFR therapy and activation of HER-3 signalling may help cells to evade the antitumour effects of anti-EGFR mAbs, such as ICR62 and/or cetuximab.

Many previous studies also suggest that MET and Src non-receptor tyrosine kinase have a role in the mechanism of resistance to anti-EGFR mAb therapies (Lu *et al*, 2007; Liska *et al*, 2011; Song *et al*, 2014). However, while we found an increase in the phosphorylation of Src in both DiFi62 and DiFiG cells compared with DiFi parental cells, we did not find significant changes in the levels of MET activation in these cells (Figure 3D). In addition, we found no significant differences in the growth of DiFi parental and DiFi62 or DiFiG cells when treated with dasatinib, a Bcr-Abl and Src dual inhibitor, or crizotinib, a cMet and Alk dual inhibitor (Figure 4A–C).

In conclusion, our results demonstrate that acquired resistance of colorectal tumour cells to treatment with anti-EGFR mAb ICR62 is accompanied by an increased level of cell surface EGFR, and upregulation of pHER-2/pHER-3. We demonstrated that the colorectal cancer cells that acquire resistance to ICR62 also acquire resistance to treatment with other anti-EGFR mAbs such as cetuximab, but remain highly sensitive to treatment with small molecule pan-HER inhibitors. Our results provide a rationale for further investigation on the therapeutic potential of the pan-HER family inhibitors in the treatment of colorectal cancer with acquired resistance to EGFR blocking antibodies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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