Global protein profiling reveals anti-EGFR monoclonal antibody 806–modulated proteins in A431 tumour xenografts

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Abbreviations:

2D DIGE - 2D difference gel electrophoresis

BVA - biological variation analysis

DIA - differential in-gel analysis

EGFR - epidermal growth factor receptor

mAb - monoclonal antibody

mAb806 - monoclonal antibody 806

PI - proliferation index

SUMMARY

An important mediator of tumorigenesis, the epidermal growth factor receptor (EGFR) is expressed in almost all non-transformed cell types, associated with tumour progression, angiogenesis, and metastasis. The significance of the EGFR as a cancer therapeutic target is underscored by the clinical development of several different classes of EGFR antagonists, including monoclonal antibodies (mAb) and tyrosine kinase inhibitors. Extensive preclinical studies have demonstrated the anti-tumour effects of mAb806 against tumour xenografts overexpressing EGFR. EGF stimulation of A431 cells induces rapid tyrosine phosphorylation of intracellular signalling proteins which regulate cell proliferation and apoptosis. Detailed understanding of the intracellular signalling pathways and components modulated by mAbs (such as mAb806) to EGFR, and other growth factor receptors, remain limited. The use of fluorescence 2D difference gel electrophoresis (2D DIGE), coupled with sensitive MS-based protein profiling in A431 tumour (epidermoid carcinoma) xenografts, in combination with mAb806, revealed proteins modulating endocytosis, cell architecture, apoptosis, cell signalling pathways and cell cycle regulation, including Dynamin-1-like protein, cofilin-1 protein, and 14-3-3 protein zeta/delta. Further, we report various proteins, including Interferon-induced protein 53 (IFI53), and Oncogene EMS1 (EMS1) which have roles in the tumour microenvironment, regulating cancer cell invasiveness, angiogenesis, and formation of metastases. These findings contribute to understanding the underlying biological processes associated with mAb806 therapy of EGFR-positive tumours, and identifying further potential protein markers that may contribute in assessment of the treatment response.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a 170 kDa membrane bound member of the type 1 receptor tyrosine kinase (RTK) family of receptors [1, 2]. Overexpression of EGFR has long been associated with many epithelial cancers, with expression correlated with disease progression and poor clinical outcome [3-5]. Overexpression of EGFR has been implicated in malignant transformation and tumour growth via apoptosis, cellular proliferation, angiogenesis and metastasis. EGFR signalling has further been shown to modulate the tumour microenvironment, regulating tumour cell synthesis and secretion of several different angiogenic growth factors, including vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) [6].

The significance of the EGFR as a therapeutic target for cancer treatment is underscored by the clinical development of a number of different classes of EGFR antagonists, including monoclonal antibodies (mAbs) and RTK inhibitors. Two mAbs that interact and bind directly to the extracellular domain of EGFR, blocking ligand binding and thereby receptor activation, have been approved for treatment of metastatic colorectal cancer; the chimeric antibody cetuximab and panitumumab [7]. Cetuximab has further been approved (Erbitux®) for the treatment of advanced head and neck cancer, in conjunction with external beam radiation therapy. However, there is significant uptake of these antibodies in normal tissues which express EGFR, including as skin and liver, which limits the administering mAb dose [8-10].

The monoclonal antibody 806 (mAb806) was raised against mouse fibroblast cells expressing the de-2-7 EGFR, a mutation of the extracellular domain of EGFR [12]. Serological and immunohistological studies have confirmed that mAb806 binds to a conformationally exposed

epitope of EGFR that is exposed in de2-7 EGFR, and when wtEGFR is overexpressed on tumour cells, but not to wtEGFR expressed on normal tissues [11]. Extensive preclinical studies have demonstrated the specific anti-tumour effects of mAb806 against tumour xenografts expressing either de2-7 or amplified EGFR, both alone and in combination with other therapies [12-16]. The specificity of mAb806 has been associated with binding to an epitope of EGFR which is exposed during conformational unfolding of wtEGFR on tumour cells prior to homo/heterodimerisation [10]. Further, the selective targeting *in vivo* of EGFR in human cancers has been confirmed in a recent clinical trial [17]. mAb806 therefore represents a selective approach to target tumour-specific growth factor receptors involved in specifically targeting the tumour progression, and cell signalling.

As a first step toward understanding the effect of mAb806-mediated anticancer activity in targeting the EGFR, we employed the use of fluorescence 2D difference gel electrophoresis (2D DIGE) [18, 19], coupled with sensitive MS-based protein profiling in A431 tumour (epidermoid carcinoma) xenografts, to monitor the effect of mAb806 on protein expression levels. In this study, we have defined conditions that enable us to examine the possible role of mAb806 in the tumour environment, specifically allowing the identification of proteins involved in apoptosis, cell signalling, cancer cell invasiveness, angiogenesis, and formation of metastases. These findings contribute to understanding the underlying biological processes associated with mAb806 therapy of EGFR-positive tumours, towards identifying further potential protein markers that may contribute in assessment of the treatment response.

EXPERIMENTAL PROCEDURES

2.1 Cell line and monoclonal antibody treatment

The A431 cell line was acquired from ATCC (Rockville, MD) and was cultured in media containing Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Islands, NY) with 10% FCS (CSL, Melbourne, Australia), 2 nM glutamine (Sigma Chemical Co, St Louis, MO) and 2 mM penicillin/streptomycin (Life Technologies, Grand Island, NY).

The murine 806 monoclonal antibody (mAb806) is a IgG2b antibody which recognises the subset of the wild type EGFR expressed on the cell surface, as previously described [10, 14, 20]. Production and purification of this antibody was performed at the Biological Production Facility (Ludwig Institute for Cancer Research Ltd, Melbourne, Australia).

2.2 Xenograft models

Approximately 1 x 10^6 A431 cells in 100 μL of PBS were subcutaneously injected into the flank of 4-6 week old female BALB/c nude mice (Animal Research Centre, Perth, Australia). Treatment was commenced when the tumour volume reached a mean of 150 mm^3 (Fig. 1). Tumour volume was calculated as (length x $width^2$)/2, where length was the longest axis and width was the perpendicular measurement [21].

Treatment consisted of either: (i) Group 1 - 100 μL vehicle (PBS); (ii) Group 2 – 1 mg/mouse mAb806 i.p., with eight mice in each group. Mice were treated with either antibody or vehicle three times a week for two weeks. Four mice were sacrificed at Day 7 after commencement of treatment (D7) to obtain tumour specimens, whilst the remaining mice were monitored for tumour growth until Day 14. This research project was approved by the Austin Health Animal

Research Ethics Committee (A2008-03115). Following sacrifice at Day 7, four tumour xenografts were removed from each experimental group and embedded in Tissue Tek Optimal Cutting Temperature Compound (Sakura Kinetek, Torrance, CA), frozen in dry ice and stored at -80°C until further use. Tumour tissue samples were used for all laboratory experiments.

2.3 Preparation of tumour xenograft homogenates

Tumour xenograft homogenates excised from four different mice per treatment group were prepared by homogenisation in RIPA buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 200 mmol/L NaO_3V, 0.4% H_2O_2, 0.5% deoxycholate, 0.005% SDS, 10 mmol/L NaF and addition of the protease inhibitor cocktail set 1) (Merck Millipore). Homogenisation was performed in a Dounce Homogeniser before incubated at 4ºC for 60 min. Samples were centrifuged at 14,000 x g for 30 min at 4ºC. The protein content of the homogenates supernatant was determined using the detergent compatible protein assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer instructions.

2.4 SDS-PAGE

Samples were prepared in 1 × Lämmli sample buffer (0.06 M Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.01 % bromophenol blue, pH 6.8) containing 50 mM DTT and heated for 5 min at 95 ºC. Proteins were separated on a 4-12 % NuPAGE® Novex Bis-Tris Gel (Invitrogen) at 150V at constant current for 1 h.

2.5 Immunoblotting

Proteins were electrotransferred onto nitrocellulose membranes using the iBlot™ Dry Blotting System (Life Technologies) and membranes blocked with 5% (w/v) skim milk powder in Tris-buffered saline with 0.05% (v/v) Tween-20 (TTBS) for 1 h. Membranes were probed with

primary anti-EGFR (Cell Signaling Technology, Danvers, MA), anti-phospho-EGFR-1173 (Invitrogen, Corporation, CA), and anti-p27/Kip1 (BD Biosciences, CA) antibodies for 1 h in TTBS (50 mM Tris, pH 7, 150 mM NaCl, 0.05% (v/v Tween 20). To ensure equal loading, immunoblots were also probed with β-actin antibody (Cell Signaling Technology, Danvers, MA). All membranes were incubated with secondary antibodies for 1 h in darkness. Western blots were scanned using the Storm 804 Phosphoimager (GE Healthcare) and analysed using the ImageQuant TL Image Analysis Software 2005 (GE Healthcare).

2.6 Preparation of tumour xenografts for Immunohistochemistry

Sections (10 μm) were cut, and fixed in 4ºC acetone for 10 min followed by air-drying for a further 10 min. Sections were washed in PBS before blocking with MAXITAGS^{TM} Protein Blocking Agent 2x (Immunon^{TM} Shandon, Pittsburgh, PA) for 10 min, prior to incubation with primary antibodies for 1 h. Antibodies used include those against EGFR (Cell Signaling Technology, Danvers, MA), phosphorylated-EGFR (p1173), Ki-67 and p27/Kip1 (Santa Cruz Biotechnology, Santa Cruz, CA). The histologic appearance of the tissue sections stained for various samples were confirmed with H&E staining. Following incubation, specimens were rinsed with washing buffer before secondary antibody was applied for 30 min. The anti-rabbit secondary antibody (Dako Group, Glostrup, Denmark) and anti-goat secondary antibody (Vector Laboratories, Burlingame, CA) were prepared according to manufacturer's specification. AEC substrate solution (0.1 mol/L acetic acid, 0.1 mol/L sodium acetate, 0.02 mol/L AEC and 0.03% H_2O_2) was used for detection. The slides were then washed and counter stained with hematoxylin (BDH Laboratory, Poole, UK) and mounted with Crystal Mount (Biomedacorp, Foster City, CA).

For Ki-67, the percentage of reactive nuclei to the Ki-67 antibody was estimated by averaging 10 representative high-power fields per treatment group. The data was then expressed as proliferation index (PI), calculated as: Ki-67 positive staining cells / total cells) x 100 [16].

2.7 TUNEL Assay

In situ DNA fragmentation assay was performed using the DACX TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labelling) assay, which detects DNA breaks using immunohistochemistry. Sections (10 μm) were cut and the assay was performed according to manufacturer's specifications (R&D Systems, Minneapolis, MN). Briefly, sections were immersed in decreasing alcohol series, followed by PBS before fixed in 3.7% Buffered Formaldehyde at room temperature (RT). Sections were labeled with 50 μL of Proteinase K solution at RT, and washed in Quenching Solution. Sections were immersed in 1x TdT Labeling Buffer, followed by incubation in 50 μL of Labeling Reaction Mix at 37°C for 1 h in a humidity chamber. The sections were immersed in 1x TdT Stop Buffer to stop the labeling reaction, and washed in PBS. Further incubation with 50 μL of diluted anti-BrdU for 1 h at 37°C was performed. Samples were washed in PBS, 0.05% Tween20 for 2 min and covered with 50 μL of Streptavidin-HRP Solution and incubated for 10 min at RT. Samples were then immersed in DAB Solution for 5 min, prior to being washed with deionized water. Samples were counterstained and prepared for viewing.

2.8 Protein extraction and quantification of tumour xenografts

Total protein was harvested from four independent tumour samples in each experimental group. Cells were dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS and 20 mM Tris-HCl. pH 8.5) immediately after sectioned by cryostat. Samples were sonicated in an ice-cold water bath for 20 min followed by centrifugation at 100,000 x g (1 h at 4°C) to

remove insoluble material. Protein concentration was determined using the 2D Quant-Kit (GE Healthcare) as per manufacturer's instructions.

2.9 2D DIGE experimental design and protein sample labelling

DIGE involves pre-separation fluorescent protein labelling with three separate dyes to reduce gel-to-gel variability, to increase sensitivity and dynamic range of protein detection, and to enhance quantification [22]. Samples were reverse labelled to minimize false positive results due to preferential dye labelling. Four independent tumour samples were prepared for each experimental group and numbered accordingly. PBS and mAb806 treated tumour samples were randomly paired for comparison. Each pair was separated on an individual gel. Protein samples were minimally labelled with fluorescent cyanine dyes for 2D DIGE analysis, according to the manufacturer's instructions (GE Healthcare). Briefly, Cy3 and Cy5 dyes (200 pmol) were used to label 25 μg of protein. Two sample pairs were reverse labelled to reduce the dye labelling bias. As per instructions, an internal standard was produced by pooling 12.5μg of each sample, 25μg of which, was combined with each labelled pair [22].

For isoelectric focussing (IEF), each dye-labelled sample was mixed with an equal volume of lysis buffer containing 40 mM DTT and 1% v/v IPG buffer, pH 3-10. Each sample pair was loaded with an internal standard and placed on IPG strips (24 cm, 3-10 linear) which had previously been hydrated overnight in DeStreak^{TM} solution containing 0.5% IPG buffer, pH 3-10. The Ettan IPGphor 3 (GE Healthcare) was used to separate the proteins by charge during IEF. This was performed over 18 h, for a total of 94,900 Vh (300V for 3 h, ramp to 1000V over 6 h, to 8000V over 3 h and hold at 8000V for 8 h.

Prior to second dimension SDS-PAGE, each strip was equilibrated in 10 mL of equilibrium buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and 0.002% w/v bromophenol blue) containing 1% w/v DTT for 15-20 min at RT, followed by second step equilibrium in the same buffer, replacing 1% DTT with 2.5% w/v iodoacetamide. IPG strips were then placed on 8-18% gradient polyacrylamide gels, cast in low-fluorescence glass plates using an Ettan-DALT caster (GE Healthcare). Second dimension gel electrophoresis was performed at 5W per gel for 30 min, followed by a constant voltage overnight (90V) at 23ºC. Voltage was stopped when the bromophenol blue dye front reached the bottom of the gel.

Protein spots were excised from the preparative 2D gels for MS analysis. This was prepared in the same manner with the gels loaded with 500 μg protein pooled from each sample. Gels were washed three times for 10 min with deionised water prior to protein visualisation using Bio-safe^{TM} CBB (Pierce, Rockford, IL). The gel with the highest number of spot features was used as the master gel for matching the remaining three gels; the spot features from mAb806-treated and PBS-treated samples were compared using both DIA and BVA modules of the DeCyder™ software.

2.10 DeCyder™ analysis and spot selection criteria

Spot map image files were analysed in batch processor mode in DeCyder™ (v5.2), which automatically processes each spot map by linking both differential in-gel analysis (DIA) and biological variation analysis (BVA) modules together [22]. Firstly, each gel was subjected to DIA, whereby spot boundaries and volumes were co-detected for Cy2, Cy3 and Cy5 spot maps on each gel. The estimated number of spots for co-detection was set to 10,000 and spot exclusion filters were applied to each gel to ensure the thorough removal of artificial spots from the analysis (*e.g.* dust particles) and other obvious non-protein spots. Spots with volume

<15,000 were excluded. Secondly, the files generated from each DIA were entered into the DeCyder™ BVA module, where each Cy2-labelled internal standard was used to match the protein spot maps from all gels. It should be noted that all identified proteins identified in these regions are reported, although without further validation the differential expression of specific (or multiple) proteins cannot be determined in these selected spots. Spot information obtained from DIA of Cy5/Cy2 and Cy3/Cy2 ratios was then normalised and used to calculate an average volume ratio for corresponding spots from each gel. The Student's *t*-test was applied to determine those protein spots that exhibited differential expression changes in response to mAb806 treatment.

2.11 Spot Excision

Selected Coomassie-stained protein spots excised and subjected to automated in-gel trypsinisation using a robotic liquid handling workstation (MassPREP™ Station, Micromass). Briefly, gel plugs were destained, reduced, alkylated (iodoacetamide), washed and dehydrated in 100% ACN. This was followed by incubation with 6 ng/mL trypsin in 50 mM ammonium bicarbonate (25 mL) for 5 h at 37°C. Peptides were extracted with 1% formic acid/2% ACN, then twice with 50% ACN. Peptide digests were concentrated by centrifugal lyophilisation (SpeedVac, Savant) to a volume of ~10 μL for nanoLC-MS/MS.

2.12 LC-MS/MS analysis

RP-HPLC was performed on a nanoAcquity® (C18) 150 × 0.15-mm i.d. reversed phase UPLC column (Waters), using an Agilent 1200 HPLC, coupled online to an LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific) [23]. The column was developed with a linear 60 min gradient with a flow rate of 0.8 μL/min at 45 °C from 0-100% solvent B where solvent A was 0.1% (v/v) aqueous formic acid and solvent B

was 0.1% (v/v) aqueous formic acid/60% acetonitrile. Survey MS scans were acquired with the resolution set to a value of 30,000. Real time recalibration was performed using a background ion from ambient air in the C-trap [24]. Up to five of selected target ions were dynamically excluded from further analysis for 3 min.

2.13 Database searching and protein identification

Parameters used to generate peak lists, using Extract-MSn as part of Bioworks 3.3.1 (Thermo Fisher Scientific), were as follows: minimum mass 700; maximum mass 5,000; grouping tolerance 0 Da; intermediate scans 200; minimum group count 1; 10 peaks minimum and TIC of 100. Peak lists for each LC-MS/MS run were merged into a single MGF file for Mascot searches. Automatic charge state recognition was used due to the high resolution survey scan (30,000). MGF files were searched using the Mascot v2.3.01 search algorithm (Matrix Science) against the Q112 LudwigNR protein sequence database with a taxonomy filter for human comprising 137 881 entries (http://www.ludwig.edu.au/archive/LudwigNR/LudwigNR.pdf). Searching parameters used were: fixed modification (carboxymethylation of cysteine; +58 Da), variable modifications (oxidation of methionine; +16 Da), three missed tryptic cleavages, 20 ppm peptide mass tolerance and 0.8 Da fragment ion mass tolerance. Significant protein identifications contained at least 2 unique peptide identifications. The false-discovery rate (derived from corresponding decoy database search [25]) was less than 1%). Pathway analysis of proteins differentially expressed in this study were assessed using Panther HMM algorithm [26, 27] and Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA; http://www.ingenuity.com). IPA was used to interpret the differentially expressed proteins in terms of predominant canonical pathways [28]. Enriched canonical pathways were identified from the IPA library

using a Fisher's exact test adjusted for multiple hypothesis testing using the Benjamini-Hochberg correction [29].

3. RESULTS AND DISCUSSION

3.1 MAb806 inhibits the growth of A431 Xenografts

BALB/c mice with A431 mice xenografts were treated with mAb806 or control (vehicle) beginning 7 days post-inoculation, when tumours were approximately 150 mm^3. Treatment with mAb806 resulted in significant tumour growth reduction, from a mean size of 1092 mm^3 in the vehicle-treated (PBS) group on day 14, compared to a mean of 623 mm^3 in the mAb806 treated group ($p=0.03$) (Fig. 1), demonstrating significant inhibition (43%) of tumour growth in response to mAb806 treatment.

3.2 MAb806 upregulates p27/Kip1, reduces proliferation, and induces apoptosis in A431 xenografts

Although the total EGFR expression and phosphorylation at Tyr1173 was similar between mAb806 treated tumours and vehicle-treated (PBS) tumours, treatment with mAb806 resulted in a significant increase in p27/Kip1 expression compared to vehicle-treated mice, with a calculated increase of 49% (Fig. 2). These results are in agreement with previous reports where treatment of xenograft tumour expressing de2–7 EGFR with mAb806 induced increased expression of p27/Kip1 (18).

Ki-67 immunohistochemical staining was also performed to determine if an anti-proliferative effect occured in conjunction with the up-regulation of p27/Kip1. The Ki-67 proliferative index was decreased in mAb806-treated compared to vehicle-treated tumours, with a mean

proliferation index (PI) of 11.52 in vehicle-treated tumours compared to 0.45 in mAb806 treated tumours (*p*-value 0.001). In addition, TUNEL staining showed an increased number of apoptotic nuclei in mAb806-treated tumours compared with PBS-treated tumours (Fig. 3), from a mean count of 1.2 to 6.0 (*p*-value 0.02) indicating increased apoptosis is induced by mAb806 treatment *in vivo*.

3.3 Comparative proteomic analysis reveals proteins associated with cell cycle regulation [30], apoptosis, angiogenesis, and metastasis modulated in response to mAb806

2D DIGE [19, 31], in combination with LC-MS/MS, was used to identify and monitor the differential expression of proteins associated with A431 tumour xenograft response to mAb806. The 2D DIGE analysis of cellular proteins is shown in Fig. 4. Over 3,000 protein spot features were detected in each gel. While using 2SD as a threshold, the expression levels of 94% of the protein spot features were similar between control and treatment groups, approximately 2.5% of proteins were significantly decreased in abundance and 3.2% of proteins increased in abundance in mAb806-treated tumours. Selected protein spot-features whose expression levels were significantly altered by mAb806 treatment were excised from the preparative gel and subsequently identified by LC-MS/MS (Supplementary Information, Table S1), as described in Experimental Procedures.

A431 tumour proteins whose expression levels were significantly increased or decreased in abundance upon mAb806 treatment, their average protein spot ratios, experimental and theoretical p*I* values, and apparent molecular masses (M_r) are listed in Table 1 (increased abundance) and Table 2 (decreased abundance). Average mAb806 treated/PBS treated ratios >1 indicate proteins that are highly expressed in mAb806 treated tumours, relative to control

PBS treated tumours while low average spot ratios (<-1) are indicative of proteins decreased in abundance as a consequence of mAb806 treatment. A comprehensive list of protein identifications is provided in Supplementary Information, Table S1.

The 3D view of selected proteins that were increased or decreased in protein expression upon mAb806 treatment, along with their protein spot volumes, are shown in Fig. 5A/B. Amongst the 49 proteins differentially expressed upon mAb806 treatment, 21 proteins exhibited ≥1.5 fold changes in their expression levels with student T-test values of p <0.05. For example, apoptosis related proteins such as Dynamin-1-like protein (-1.6-1.9), cofilin-1 (+3.3-3.5), cortactin (+1.3), 14-3-3 protein zeta/delta (-2.5), apoptosis-linked gene-2 (ALG-2) (+1.3), and Interferon-induced protein 53 (+1.3-1.4) were clearly dysregulated in response to mAb806. Pathway analyses for these data were performed using the Panther HMM algorithm and Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc.). For all proteins identified in this study, pathways associated with cadherin signalling (6.8%), apoptotic signalling (5.1%) were identified. Further, endocrine system development and function, drug / lipid metabolism, molecular transport, epithelial junction remodelling, and clathrin-mediated endocytosis were shown to be enriched in this study (p<0.05). For proteins identified with increased abundance (Table 1), using Panther HMM key pathways associated with EGF receptor signalling, FAS signalling, G-protein signalling, and chemokine and cytokine signalling pathways, while for Ingenuity Pathway Analysis, cellular assembly and organisation, cellular function and maintenance, and DNA replication, recombination, and repair, and energy production were shown to be functions associated with these data. For Table 2 (decreased abundance), PDGF signalling, Ubiquitin proteasome pathway, Glycolysis, and Rho GTPase cytoskeletal regulation were highlighted using Panther HMM key pathways. Further, Ingenuity Pathway Analysis

showed cell death and survival, RhoGDI signalling, clathrin-mediated endocytosis, and lipid metabolism associated with proteins identified enriched in this study (Table 2).

Here, we demonstrate that treatment with mAb806 in A431 xenografts resulted in inhibition of tumour growth, and significantly elevated expression of p27/KIP1, the latter of which has not been previously described in this model. To assess the mechanism of action of mAb806 *in vivo*, we have used global protein profiling with 2D DIGE and sensitive MS-based protein profiling in A431 tumour xenografts to assess protein expression changes in response to mAb intervention. The most significantly dysregulated proteins have previously been associated with regulating cell structure, apoptosis, cell signalling pathways, endocytosis/internalisation, cell invasiveness, and angiogenesis. Subsequent TUNEL assay analyses of these samples confirmed the presence of elevated apoptosis in the treated samples and reduction of proliferative index on Ki-67 immunohistochemistry staining also confirming the anti-proliferative effect of mAb806 treatment.

Cofilin (3.3-3.5) was increased in abundance by mAb806 treatment, and is a protein in the actin-depolymerizing family known to be essential for the dynamic changes in the actin cytoskeleton, which occurs during cell locomotion or other processes that are accompanied by a reorganisation of the cellular shape. Cofilin activity is regulated by phosphorylation of a crucial serine residue (serine-3), which results in inactivation of its depolymerisation activity [32]. LIM kinase 1 (LIMK1) regulates actin dynamics by inhibiting cofilin [33, 34]. LIMK1-cofilin signalling pathway has a pivotal role on the efficient translocation of EGFR out of the early endosomes to the late endosomes through regulating the actin cytoskeleton. As EGFR signalling occurs principally following endocytosis, the changes in cofilin levels may contribute to altered signalling as a result of mAb806 treatment.

14-3-3 proteins are a ubiquitous group of proteins which have been implicated in a variety of cellular activities, including cell cycle regulation [30], apoptosis, neurotransmitter synthesis, neuronal development, and exocytosis [35, 36]. 14-3-3 proteins have been shown to interact with enzymes involved in cell signalling transduction pathways. The 14-3-3ζ isoform is one of 7 isoforms found in mammals, which binds to several different enzymes and may reduce apoptosis. In the case of 14-3-3ζ, it has been shown that a slight increase in the protein expression level of the isoform is sufficient to induce tumorigenesis in mice [37]. Knockdown of 14-3-3ζ has been shown to sensitize cells to stress- and anoikis- induced apoptosis [38, 39]. The 14-3-3ζ down-regulated cells were shown to increase activation of JNK and p38 proteins, two key proteins in apoptotic signal transduction [38]. In response to EGF-stimulation, the direct interaction 14-3-3ζ with EGFR forms a 14-3-3-EGFR complex, which has a role in the Ras-Raf-MEK-MAPK signalling pathway [40]. In addition, interactions between 14-3-3ζ and cofilin and LIMK1 (cofilin key regulator) indicate that the 14-3-3 proteins affect the microfilament cell structure [41-43]. In our study, a 2.5 fold reduction of 14-3-3ζ protein expression was observed in A431 xenograft tumours treated with mAb806. Considering the function and oncogenic character of 14-3-3ζ, it is likely that reduction in 14-3-3ζ expression is an important event in the anti-tumour effects of mAb806 against tumour xenografts overexpressing EGFR.

Further, Oncogene EMS1 (Cortactin) was shown to increase abundance 1.3 fold following mAb806 treatment. EMS1 contributes to the organization of the actin cytoskeleton and cell structure, playing an important role in regulation of cell migration, cancer cell invasiveness, and the formation of metastases (41). Cortactin has further involved in endocytosis and actin cytoskeletal function, reported as an actin-associated scaffolding protein, which binds and

activates the actin-related protein 2/3 complex, regulating the branched actin networks in the formation of dynamic cortical actin–associated structures (42). We have previously shown that mAb806 binding to EGFR causes prompt internalisation of mAb806:EGFR complexes, and this intracellular trafficking is via a dynamin-regulated process (43). Therefore, changes in cortactin and cofilin levels would be consistent with activated processing of mAb806:EGFR complexes in treated A431 tumours. Interestingly, dynamin levels were reduced in mAb806-treated tumours, which may reflect a feedback-loop down-regulation of dynamin as a consequence of the mAb806:EGFR internalisation process. Dynamin has also been shown to interact with the SH3 domain of cortactin, which may provide a further link between endocytosis of the EGFR complex and actin cytoskeletal function (44).

In addition, we observed increased abundance of Interferon-induced protein 53 (IFI53) (+1.3-1.4) in response to mAb806. IFI53 (tryptophanyl-tRNA synthetase) has been demonstrated to regulate ERK, Akt, and eNOS activation pathways that are associated with angiogenesis, cytoskeletal reorganization and shear stress-responsive gene expression (45). Secretion of IFI53 is mediated by the dissociation with Annexin II in the cytosol (46). Interestingly, cytosolic Annexin II (P07355) was observed to be decreased in abundance (-1.5) in response to mAb806 treatment in this study. Like other aminoacyl-tRNA synthetases, IFI53 controls angiogenesis and immune responses and that may have roles throughout the tumour microenvironment (47). At present, the role of IFI53 in response to mAb cancer therapy remains unclear, and requires further investigation.

4. CONCLUSION

The use of global protein profiling provides a unique approach to identify components which are dysregulated in expression following exposure to mAb806. This targeted, *in vivo* study permitted the identification of a potential cluster of functional proteins that may be regulated by downstream pathways. These approaches will allow more accurate assessment of the functional effects of antibody-receptor binding interactions, and assist with the understanding of the effects of this therapeutic approach. The findings presented in this study contribute to understanding the underlying biological processes associated with mAb806 therapy of EGFR-positive tumours, highlighting proteins involved in endocytosis, cell architecture, apoptosis, cell signalling, cancer cell invasiveness, angiogenesis, and metastases. These findings also provide a tool for identifying potential markers that may contribute in assessment of mAb 806 treatment responses.

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Declaration of Interest

The authors declare no conflict of interest

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Figure Legends

Figure 1 - Tumour growth curve of A431 xenografts treated with mAb806 (dotted line) vs. vehicle control (solid line). Mice were treated 3 times a week for 2 weeks (arrows).

Figure 2 - Immunoblot of A431 xenografts treated with mAb806 and vehicle control (PBS).

Figure 3 - TUNEL Assay showing an increased number of apoptotic nuclei (arrows) in mAb806-treated tumours (B) compared with PBS-treated tumours (A).

Figure 4 - 2D DIGE analysis of cellular proteins from PBS and mAb806 treated A431 xenografts. Three Cy-dye labelled extracts (25μg protein) were combined and separated by 2-DE (see Experimental Procedures). Protein spots decreased in abundance upon mAb806 treatment are shown in green colour whereas increased protein abundance protein spots are shown in red. Protein spots are numbered and MS-based identifications are listed in the Supplementary Information, Table S1; proteins increased or decreased in abundance following mAb806 treatment are listed in Tables 1 and 2.

Figure 5 - Representative 3-D views and the graph views of protein spot volumes of secreted proteins increased (panel A) or decreased (panel B) in abundance upon mAb806 treatment. Data were obtained from 2-D DIGE shown in Fig. 4 and analysed using the 'DeCyder' software described in Section 2. The 3-D views (left side) shown are from one representative gel and the statistical analysis (right side), along with p-values, was performed using all gels. Protein spot numbers are listed in Tables 1 and 2, as well as Table S1 of Supporting Information.

Table 1 - Proteins increased in abundance in mAb806 treated xenografts.

Table 2 - Proteins decreased in abundance in mAb806 treated xenografts.

Table S1 (Supplementary Information) - Comprehensive list of MS-identified protein spots, along with their molecular masses, p*I* values and fold protein expression changes.