Mutation specific functions of EGFR result in a mutation-specific downstream pathway activation

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ABSTRACT

Background

EGFR is frequently mutated in various types of cancer. Although all oncogenic mutations are considered activating, different tumor types have different mutation spectra. It is possible that functional differences underlie this tumor-type specific mutation spectrum.

Methods

We have determined whether specific mutations in EGFR (EGFR, EGFRvIII and EGFR-L858R) have differences in binding partners, differences in downstream pathway activation (gene expression and phosphoproteins), and have functional consequences on cellular growth and migration.

Results

Using biotin pulldown and subsequent mass spectrometry we were able to detect mutation specific binding partners for EGFR. Differential binding was confirmed using a proximity ligation assay and/or Western Blot for the dedicator of cytokinesis 4 (DOCK4), UDP-glucose glycoprotein glucosyltransferase 1 (UGGT1), MYC binding protein 2 (MYCBP2) and Smoothelin (SMTN). We also demonstrate that each mutation induces the expression of a specific set of genes, and that each mutation is associated with specific phosphorylation patterns. Finally, we demonstrate using stably expressing cell lines that EGFRvIII and EGFL858R display reduced growth and migration compared to EGFR wildtype expressing cells.

Conclusion

Our results indicate that there are distinct functional differences between different EGFR mutations. The functional differences between different mutations argue for the development of mutation specific targeted therapies.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is a member of the ERBB protein family and is localised on the cell membrane. The receptor is activated by members of the pidermal growth factor (EGF) family (a.o. EGF, amphiregulin, TGF-a, HB-EGF and epiregulin) and binding of one such ligand results in receptor dimerisation which induces receptor phosphorylation, recruitment of adaptor proteins and subsequent activation of signal transduction cascades [1,2].

Somatic mutations in the EGFR gene are found in several types of cancer and the mutation spectrum includes gene amplifications, gene-fusions, deletions in the extracellular domain (e.g. EGFRvIII; a deletion of exons 2–7), deletions in the intracellular domain (e.g. EGFRvV; a deletion of exons 25–28) and mutations affecting the tyrosine kinase domain (mainly exon 19 and codon L858) [3–5]. These mutations result in a constitutively activated isoform of the protein and contribute to oncogenic transformation [5–8].

Although EGFR mutations are activating, there are marked differences in the spectrum of mutations between tumour types. For example, the c.2573T > G missense mutation, resulting in the L858R substitution, is found in _10–15 % of all pulmonary adenocarcinomas [4]. This mutation is the most frequent of all mutations in EGFR but has thus far never been identified in glioblastomas (GBMs) [3]. The most common mutations in GBMs affect the extracellular domain of EGFR, including EGFRvIII (~ 30 % of all GBMs) and the A289V and V598V missense mutations [3]. These extracellular domain mutations are not found in pulmonary adenocarcinomas [4]. One of the explanations for these tumour-type specific mutations is that each mutation invokes a unique signal transduction cascade. Indeed, EGFRvIII and EGFRwt have differential activation of the JNK, STAT and MAPK signalling pathways and induce the expression of a unique set of genes [9–13]. Because different tumour types may be dependent on the unique pathways that are activated by different EGFR mutations, studying these functional differences between mutations may identify novel, tumour type specific treatment targets. Here, we have further evaluated differential activation of signal transduction pathways by EGFR-wt, EGFR-L858R and EGFRvIII.

MATERIALS AND METHOD

EGFRvIII and EGFR-L858R cDNAs were obtained from Addgene (Cambridge, MA), EGFR wildtype was a gift from Ton van Agthoven and cloned into pcDNA3.1/CT-

GFP-TOPO (Invitrogen, Bleiswijk, the Netherlands). A biotin tag and eGFP were inserted C-terminal to the transmembrane domain of EGFR to retain the integrity of the C-terminal (intracellular) domain of EGFR. To demonstrate functionality, we transfected EGFRbio-GFP into ZR-75-1 cells. Normally, ZR-75-1 cells do not proliferate in the presence of tamoxifen [43]. However, ZR-75-1 cells expressing EGFR-bioGFP, cultured in the presence of tamoxifen, responded to EGF stimulation by an increase in cell proliferation, demonstrating the construct remained functional (not shown). Stably transfected HOG (human oligodendroglioma cells [44]) cell lines were created by transfection, geneticin selection and FACS sorting. Stable cell lines were derived from bulk culture and not form a single sorted cell followed by clonal propagation.

Migration and proliferation assays were performed using an Incucyte (Essen Bioscience, Ann Arbor, MI). For proliferation experiments, 50,000 cells/well were plated in a 24-well Greiner plate (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Growth curves were constructed using the Confluence v1.5 metric of the Incucyte software. For migration experiments, cells were grown to confluence in a 24-well Essen ImageLock-plate after which a cell-free zone (scratch) was created using a WoundMaker. Wells were then cultured in serum-free media.

Constructs containing EGFRwt-BG, EGFRvIII-BG and EGFRL858R-BG were transfected into HEK cell lines using Polyethylenimine 'Max' (Polysciences, Eppelheim, Germany). The EGFRwt-BG, EGFRvIII-BG and EGFRL858R-BG proteins were then isolated using Dynabeads (Life Technologies, Carlsbad, CA, United States of America (USA)) as described previously [39]. Purified proteins were washed and loaded on a SDS page gel. Nanoflow LC-MS/MS analysis was performed essentially as described by van den Berg et al. [45]. Candidate binding proteins that were present in a GFP control pulldown or identified in $> 10\,\%$ of CRAPome experiments were omitted from the analysis [14]. We focused on candidate binding proteins that were identified with MASCOT scores > 300.

Western blots were performed as described [39]. Antibodies used were DOCK4 (1:100), UGGT1 (1:100), DDX21 (1:500) all from Sigma–Aldrich (Zwijndrecht, the Netherlands), EGFR (1:1000, Cell Signaling, Boston, MA) and GFP (1:5000, Abcam, Cambridge, United Kingdom (UK)).

Cells (HOG, U87MG, HEK) were cultured on glass slides for immunocytochemistry. Glioma samples were obtained from the Erasmus MC glioma tissue bank. Use of patient material for current study was approved by the Institutional Review Board. Antibodies used for immunocytochemistry and/or proximity ligation assays were

EGFR (1:200, DAKO, Heverlee, Belgium) and DOCK4 (1:100), MYCBP2 (1:200) and SMTN1 (1:100) all from Abcam. Proximity ligation assays were performed using a Duolink (Sigma–Aldrich) kit according to the manufacturer's instructions.

HEK cells were transiently transfected with EGFRbioGFP, EGFRL858R-bioGFP and EGFRvIII-bioGFP or BIO-eGFP constructs. Twenty hours after transfection, cells were FACS sorted to select for eGFP expressing cells. Cells were then snap frozen in liquid nitrogen and stored at _80 _C. RNA extraction was performed using TriZol (Invitrogen) and checked for RNA quality on a Bioanalyzer (Agilent, Amstelveen, the Netherlands). Gene expression was performed using HU133 plus2 arrays (Affymetrix, High Wycombe, UK) run by AROS Applied Biotechnology (Aarhus, Denmark). All experiments were performed in triplicate with each replicate experiment performed on separate days.

For reversed phase protein array (RPPA) analysis, stably transfected HOG cells were plated in six well plates and incubated in serum supplemented medium, or serum depleted medium (24 h depletion) ± 200 ng EGF for 5 min. RPPA arrays were performed by the MD Anderson RPPA core facility. Luciferase activity was measured by Dual–Luciferase Reporter Assay System (Promega). Pathway analysis was performed using Ingenuity (Redwood City, CA) and David [46].

RESULTS

We first generated constructs of wildtype EGFR and of two common mutations, EGFRvIII and L858R, and inserted a biotinylation tag and eGFP in-frame and Cterminal to the transmembrane domain. Constructs are referred to as EGFRwt-BG, EGFRvIII-BG and EGFRL858R-BG respectively. Mass spectrometry following pulldown of biotinylated constructs identified over 3000 candidate binding partners for at least one of these constructs. When filtering for duplicate hits, and removal of proteins identified by a bio-eGFP control pulldown or present in > 10 % of crapome pulldown experiments [14], our list of candidate EGFR binding proteins included 87 unique proteins (Supplementary Table 1). Almost half (37/87) of these binding partners are known interactors of EGFR and include CBL, PIK3CA, PIK3R3, SHC1 and SOS1 [15–17].

Ingenuity pathway analysis indicated that the candidate EGFR associated proteins are involved in EGF signaling and Clathrin mediated endocytosis signalling. Candidate EGFR interacting proteins are enriched for proteins that are somatically mutated in GBMs. For example, 7/87 (8.0 %) genes are mutated at a population frequency > 1.5 %

(i.e. mutations found in at least 5/290 tumours) in the TCGA, a 3–4-fold enrichment compared to all genes mutated at this frequency in GBMs (485/_20.000 genes, 2.4 %, P = 0.007, Fishers' exact test).

Of the 87 candidate binding proteins, 22 showed selective association to one of the EGFR constructs (Table 1). Selective association was defined as a relative difference in mascot scores > 3, and an absolute difference in mascot scores > 500 between any of the three constructs. The strongest candidate proteins included DOCK4 (dedicator of cytokinesis 4) UGGT1 (UDPglucose glycoprotein glucosyltransferase 1), MYCBP2 (MYC binding protein 2, E3 ubiquitin protein ligase) and SMTN (Smoothelin).

Table 1. Proteins showing selective binding to one or more specific EGFR mutations

symbol	EGFR wt	EGFR V111	EGFR-p.L858R
AP1B1		961	
CRKL	561	740	229
DNM2	462	798	211
DOCK4	2725	2136	536
EXOC7	533	213	
IFI16	772	1079	
IPO5	1403	411	217
LAD1	106	669	1056
LMO7		340	1362
MYCBP2		1606	
MYO1E		156	503
NGLY1	353	1310	887
PIK3CA	147	902	86
PIK3CB	474	828	72
RTCB		558	341
SEL1L		604	174
SMTN			546
SPECC1L		69	909
SVIL		160	741
TNPO2	410	515	
UBE3C	157	828	280
UGGT1		1573	

Western blots on independent biotin pulldowns confirmed that DOCK4 binds preferentially to EGFRvIII-BG and EGFRwt-BG but not to EGFRL858R-BG (Fig. 1b, Western blot experiments in two independent experiments). The association was further confirmed using a proximity ligation assay (PLA, Fig. 1c). OCK4 also associates with

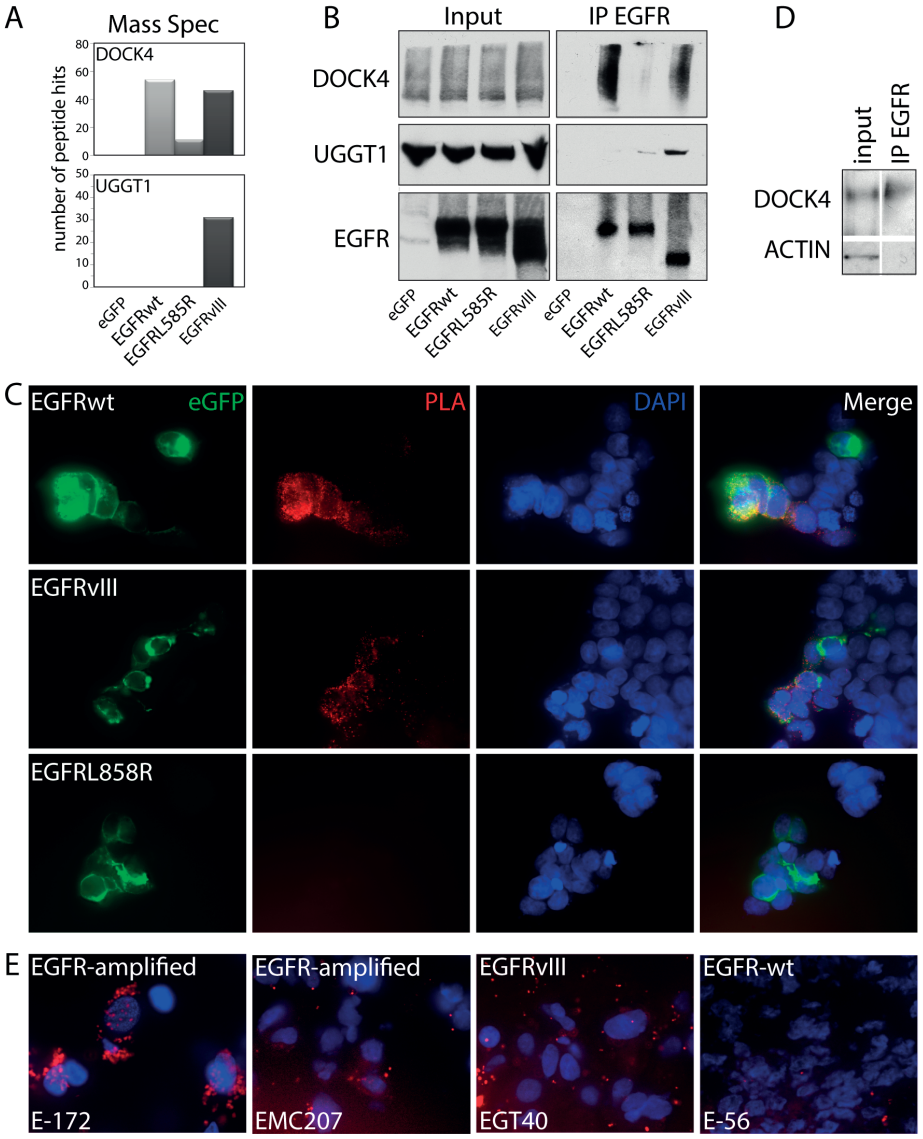

Figure 1. EGFRwt-BG, EGFRvIII-BG or EGFRL858R-BG associate with specific proteins. A) mass spectrometry results for DOCK4 and UGGT1 showing differential binding to EGFR mutations. B) Confirmation of the mass spectrometry results by Western Blot on an independent pulldown. C) A proximity ligation assay confirms that DOCK4 colocalizes with EGFRwt-BG and EGFRvIII-BG but not with EGFRL858R-BG or Bio-GFP control (not shown). All images taken at 63x magnification. D) Native EGFR also associates with DOCK4 as determined by immunoprecipitation of EGFR. E) A proximity ligation assay shows that DOCK4 and EGFR also colocalize in tumors.

EGFR also under native conditions as demonstrated by a co-immunoprecipitation using anti-EGFR antibodies in non-transfected HEK cells (Fig. 1d). Furthermore, PLA confirmed that DOCK4 and EGFR are also colocalised in EGFR amplified GBMs (Fig.

1e). DOCK4 remains associated with EGFR-wt in cells that were serum starved overnight followed by EGF stimulation (Supplementary Fig. 1). These data demonstrate that DOCK4 associates with EGFRwt-BG and EGFRvIII-BG and not (or to a lesser extent) with EGFRL858R-BG.

Because DOCK4 has been implicated in wnt pathway activation [18] we screened for differential ctivation of this pathway activation by the different EGFR constructs. However, both under basal and under wnt activated conditions, no differences in wnt pathway activation were identified (n = 3 independent experiments, data not shown).

Mass spectrometry also highlighted that UGGT1 and MYCBP2 preferentially associate with EGFRvIIIBG and that SMTN preferentially associates with EGFRL858R-BG. PLA assays confirmed the association for all three proteins (Fig. 2), Western blot (WB) further confirmed the association of UGGT1 with EGFRvIII-BG (Fig. 1b). It should be noted that some (minor) association of UGGT1, MYCBP1 and SMTN to other EGFR constructs were found by PLA and/or WB.

We hypothesised that the selective association of different EGFR mutations ultimately would result in the induction of a unique set of genes. We have therefore performed gene expression profiling of cells expressing EGFRwt-BG, EGFRvIII-BG, EGFRL858R-BG or BIOeGFP constructs (n = 3 per construct). Statistical analysis of microarrays (SAM) identified 74, 109 and 187 probesets that were differentially expressed in EGFRwt- BG, EGFRvIII-BG and EGFRL858R-BG expressing cell lines compared to BIO-eGFP control (with differential expression >2 and at a false discovery rate (fdr) <0.05, Supplementary Table 2). These probesets correspond to 61, 89 and 156 genes respectively. Many of these genes are found in all three comparisons and are involved in the transcription of DNA and are significantly enriched for the gene-ontology (GO) terms ‘sequence-specific DNA binding’, ‘transcription factor activity’, ‘transcription regulator activity’, ‘DNA binding’ and ‘protein dimerization activity’ (all $P < 0.001$). Top networks identified by Ingenuity pathway analysis include ‘Cellular compromise, cellular function and maintenance, gene expression’, ‘developmental disorder, hereditary disorder, neurological disease’ and ‘neurological disease, cell-mediated immune response, cellular development’.

To determine whether specific mutations have specific gene-expression signatures, we performed SAM analysis comparing gene expression between the different EGFR mutations. A total of 17, 12 and 35 probesets were identified that were differentially expressed between EGFRwt-BG versus EGFRvIII-BG, EGFRwt-BG versus EGFRL858R-BG and EGFRvIII-BG versus EGFRL858RBG respectively (with differential

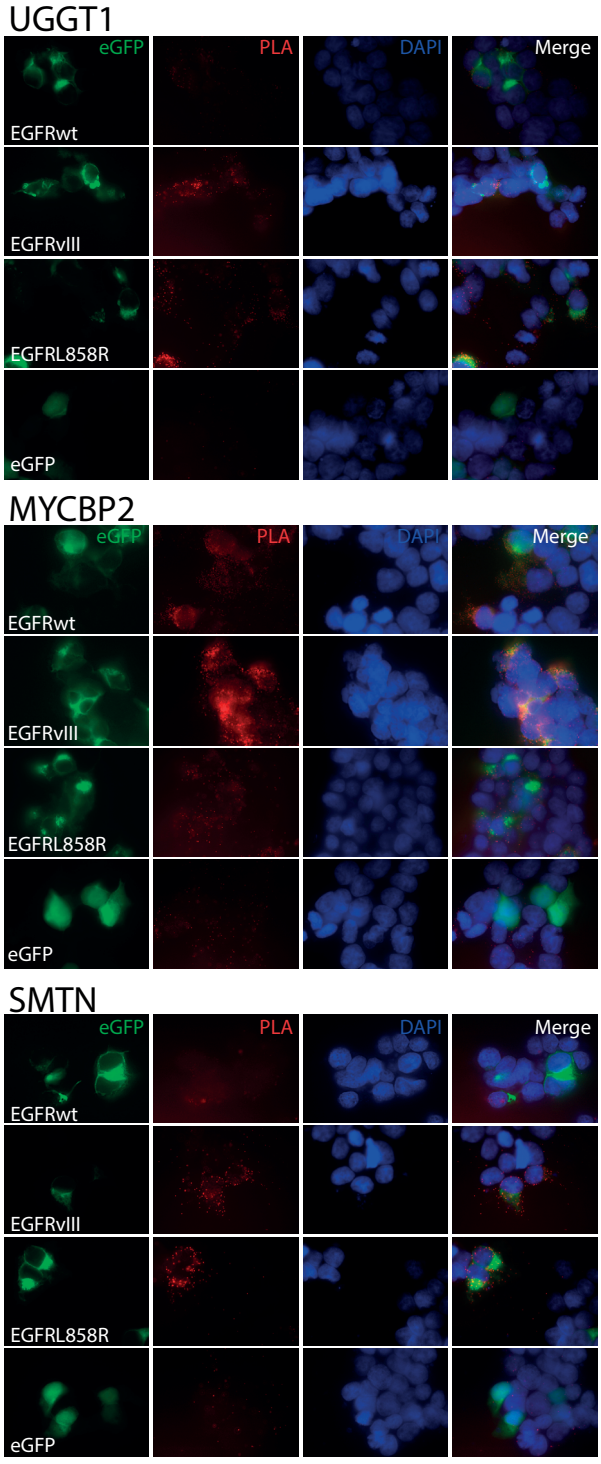

Figure 2. EGFRwt-BG, EGFRvIII-BG or EGFRL858R-BG associate with specific proteins. A proximity ligation assay shows that UGGT1 colocalizes with EGFRwt-BG and EGFRvIII-BG but not with EGFRL858R-BG or Bio-GFP control (see also figure 1B). Similarly, MYCBP2 colocalizes predominantly with EGFRvIII-BG wheras SMTN predominantly colocalizes with EGFRL858R-BG. All images taken at 63x magnification.

Table 2. Probesets that are differentially expressed between $EGFR^{wt\text{-}BG}$ *v.* $EGFR^{vIII\text{-}BG}$, $EGFR^{wt\text{-}BG}$ *v.* $EGFR^{L858R\text{-}BG}$, and $EGFR^{vIII\text{-}BG}$ *v.* $EGFR^{L858R\text{-}BG}$ as identified by SAM analysis

Gene_Symbol	Probeset_ID	eGFP	EGFRwt	EGFRvIII	EGFR-p. L858R	wt v vIII	wt v L858R	vIII v L858R
SOCS3	227697_at	4.5	4.9	9.1	5.6	X		X
RAP1A	1555339_at	13.4	4.9	7.0	4.7	X		X
C10orf10	209183_s_at	6.6	6.8	8.9	6.8	X		X
Hs.527973	206359_at	4.3	4.6	6.6	4.9	X		X
RAP1A	1555340_x_at	14.3	5.5	7.3	5.1	X		X
XR_132893	1565830_at	6.0	4.6	6.0	5.0	X		
SOCS2	203372_s_at	6.8	6.9	7.9	7.4	X		
WDR78	1554140_at	6.5	5.1	6.5	5.7	X		
DTX3L	225415_at	6.6	6.5	7.4	6.6	X		
BC042589	235456_at	7.8	6.4	7.4	6.9	X		
KIAA1267	224489_at	6.8	6.0	6.9	6.4	X		
RAB30	229072_at	6.1	4.9	6.0	5.1	X		
AK022645	232257_s_at	5.2	4.1	5.2	4.5	X		
HSPA6	213418_at	4.8	8.2	6.7	9.3	X		X
EGR1	201693_s_at	6.0	8.9	7.7	10.9	X		X
EGR1	201694_s_at	8.2	11.2	10.2	12.6	X		X
EGFR	210984_x_at	5.6	10.0	9.0	10.8	X		
AKIRIN2	223143_s_at	5.1	5.0	5.8	6.1		X	
ARC	210090_at	5.8	6.8	6.3	9.3		X	
ARL5B	242727_at	5.9	6.1	6.0	7.3		X	X
CCNA1	205899_at	3.9	4.5	3.9	5.9		X	X
EGR3	206115_at	5.6	7.1	6.1	9.3		X	X
FOS	209189_at	5.2	7.1	7.2	9.1		X	
IL12A	207160_at	5.4	5.7	5.8	6.9		X	
PHLDA1	217997_at	4.6	4.6	4.6	5.9		X	
SGMS2	242963_at	5.1	5.1	5.1	6.5		X	
TAC1	206552_s_at	4.8	6.7	6.0	8.2		X	X
TFPI2	209277_at	3.6	4.1	3.6	6.3		X	X
TFPI2	209278_s_at	5.4	6.6	5.6	8.7		X	X
HSPA1L	210189_at	7.5	8.2	7.4	8.7			X
HSPH1	208744_x_at	9.4	9.7	9.2	10.3			X
DNAJB1	200666_s_at	9.4	10.8	9.9	11.8			X
LOC100652898	227404_s_at	6.5	9.5	8.4	10.7			X
INSIG1	201627_s_at	11.0	11.5	10.6	11.7			X
DUSP6	208891_at	6.0	8.1	7.6	8.6			X
DNAJB1	200664_s_at	8.2	9.6	8.7	10.7			X
ANXA1	201012_at	6.1	6.8	6.6	7.8			X

Table 2. Probesets that are differentially expressed between $EGFR^{wt\text{-}BG}$ *v.* $EGFR^{vIII\text{-}BG}$, $EGFR^{wt\text{-}BG}$ *v.* $EGFR^{L858R\text{-}BG}$, and $EGFR^{vIII\text{-}BG}$ *v.* $EGFR^{L858R\text{-}BG}$ as identified by SAM analysis (continued)

Gene_Symbol	Probeset_ID	eGFP	EGFRwt	EGFRvIII	EGFR-p. L858R	wt v vIII	wt v L858R	vIII v L858R
KCTD12	212188_at	8.9	9.7	8.9	10.7			X
HSPA6	117_at	5.4	7.0	6.0	7.9			X
DUSP6	208892_s_at	5.1	7.3	6.8	7.9			X
KCTD12	212192_at	9.8	10.2	9.7	10.8			X
ZCCHC12	228715_at	9.0	10.0	9.0	10.9			X
ETV5	203349_s_at	5.5	8.6	8.0	9.3			X
EGR2	205249_at	6.1	7.6	6.8	9.4			X
C11orf96	227099_s_at	8.5	10.3	9.5	12.0			X
GPR50	208311_at	7.2	8.0	7.2	9.1			X
DOK5	214844_s_at	4.4	5.1	4.5	6.0			X
INSIG1	201625_s_at	9.2	9.8	9.0	10.2			X

Differentially expressed genes (>2 fold change in expression level, fdr <0.2) between mutations are marked with X in one of the last three columns.

expression > 2 and fdr < 0.2, Table 2, Fig. 3). These probesets correspond to 15, 11 and 26 different genes respectively. Genes specifically induced by EGFRvIII-BG expression include SOCS3, C10ORF10 and DTX3L (_10, 4, and 2-fold induction respectively). EGFRL858R-BG specifically induces the expression of ARC, TFPI2, SGMS2, ARLB5 and CCNA1 (~ 8, 8, 3, 2 and 4-fold respectively). Gene expression analysis therefore indicates that different mutations in EGFR induce the expression of a unique set of genes.

We next analysed phosphoprotein levels by RPPA arrays on HOG cells stably expressing EGFRwt-BG, EGFRvIII-BG, EGFRL858R-BG or Bio-eGFP control. Three conditions were examined: normal (serum supplemented cell culture), serum free and serum free, EGF stimulated. All data are listed in Supplementary Table 3. Analysis of EGFR on these arrays demonstrates that all stably transfected cell lines, apart from the Bio-eGFP control, have increased levels of EGFR and show increased EGFR phosphorylation on pY1068 and pY1173. Serum deprivation does not result in a loss of EGFR phosphorylation (pY1068 and pY1173) which suggests that EGFR signalling remains active under these conditions. Finally, EGF stimulation results in a strong increase in EGFR_pY1068 (and to a lesser extent in pY1173), predominantly in EGFRwt-BG and EGFRL858R-BG expressing cells but also in BIOeGFP and expressing cells. EGF stimulation does not activate EGFRvIII-BG which is in-line with the fact that this mutation affects the EGF binding domain.

Figure 3. Genes that are differentially expressed between EGFRwt-BG *v.* EGFRvIII-BG, EGFRwt-BG *v.* EGFRL858R-BG, and EGFRvIII-BG *v.* EGFRL858R-BG as identified by SAM analysis. Bio-eGFP control is included for reference. Scales are color coded from 13.0 (red), 7.0 (grey) to 4.5 (blue) as RMA expression values.

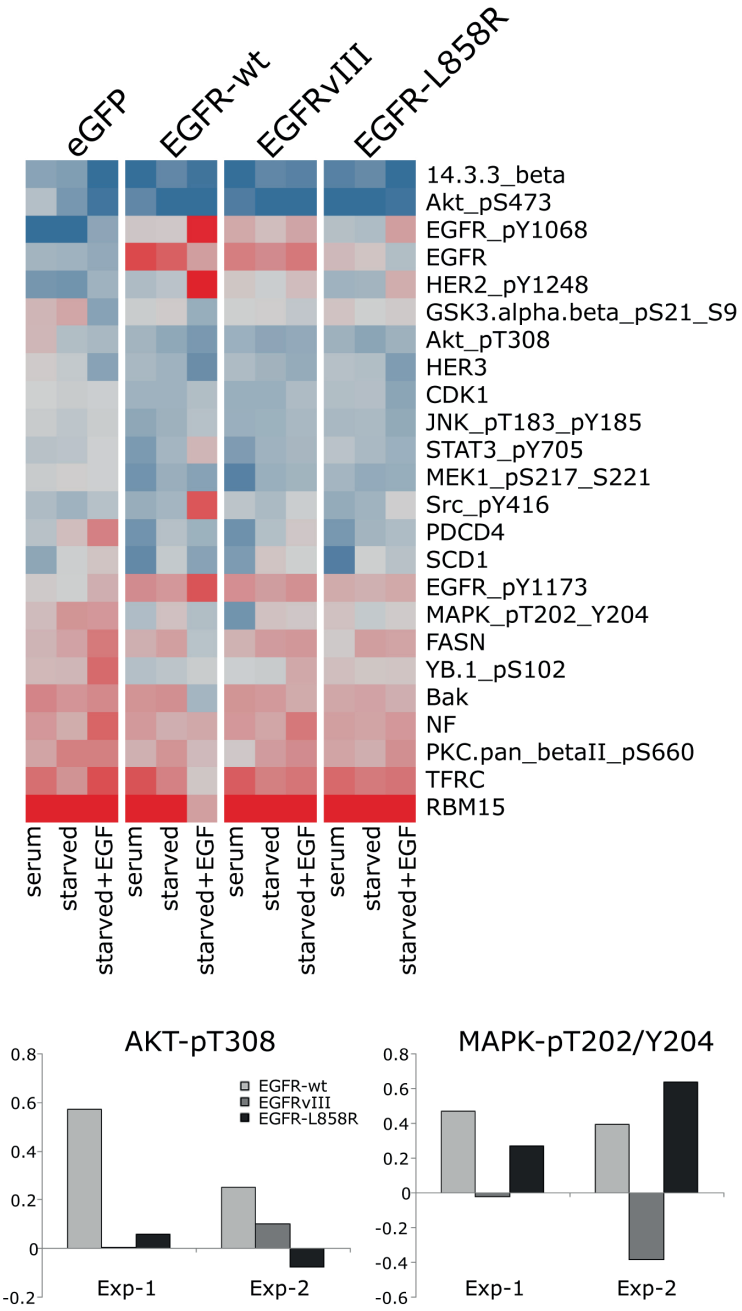

Figure 4. Proteins with >2 fold change in levels between different constructs as determined by RPPA analysis. Shown are RPPA results of these proteins in cells expressing EGFRwt-BG, EGFRvIII-BG, EGFRL858R-BG or Bio-eGFP control under normal cell culture conditions (serum supplemented) and serum free cultures ± EGF. Colors are scaled from the minimum value (blue, -1.26), average (grey, 0.41) to max RPPA vaule (red, 3.42). B) Confirmation by an independent RPPA experiment of AKT-pT308 (left) and MAPK_pT202 (right). Results of the original (exp-1) and confirmation (exp-2) are shown.

To determine whether specific mutations induce differences in their downstream pathway activation, we screened all proteins that showed a >2-fold change in levels between different constructs (Fig. 4). Examples of differences identified include, under serum conditions (i) lower levels of AKT-pT308 (and AKT_pS473) in EGFRL858R-BG expressing HOG cells compared to EGFRwt-BG, EGFRvIII-BG or BIO-eGFP expressing cells; (ii) lower levels of MAPK_pT202 phosphorylation in EGFRvIII-BG expressing cells compared to those expressing EGFRwt-BG and EGFRL858R-BG. Virtually identical data were obtained in an independent RPPA experiment (Fig. 4, and Supplementary Fig. 2). Western blot experiments (independently performed) further confirmed the differences in AKT-pT308 and MAPK_pT202 phosphorylation (Fig. 4). These data therefore indicate that different mutations in EGFR can induce a differential downstream pathway phosphorylation.

Because our results indicate that each mutation has unique molecular properties, we determined whether the various forms of EGFR also differentially affect cell physiology. HOG cells stably expressing EGFRvIII-BG and EGFRL858R-BG showed a decreased proliferation compared to bio-eGFP or EGFRwt-BG expressing cells (Fig. 5). The differences between constructs were consistently observed over multiple experiments (n = 4 experiments, six wells/experiment and four locations/well). In a wound healing

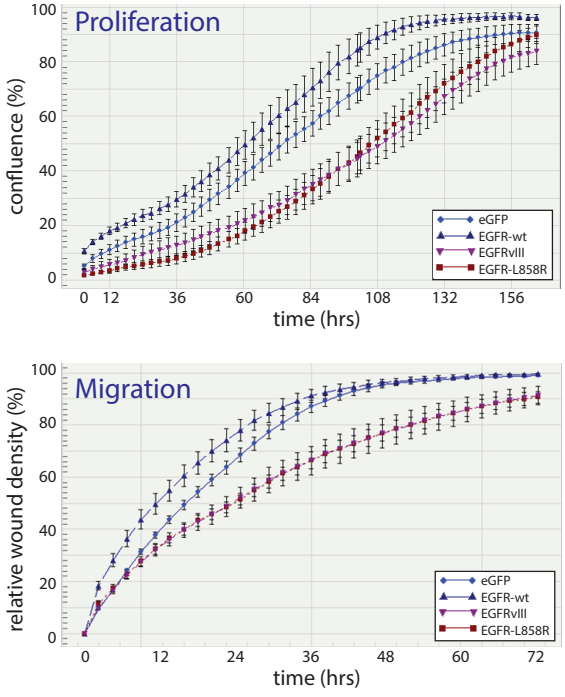

Figure 5. Mutations in EGFR differentially affect proliferation (top) and migration (bottom) in HOG cells stably transfected with EGFRwt-BG, EGFRvIII-BG, EGFRL858R-BG or Bio-eGFP control. EGFRvIII-BG and EGFRL858R-BG have virtually identical migration.

assay, the EGFRvIII-BG and EGFRL858R-BG expressing HOG cells also had a significantly slower migration compared to bio-eGFP or EGFRwt-BG expressing cells ($P < 0.001$, for all comparisons Fig. 5). The difference between constructs was consistently observed in two independent experiments ($n = 2$ experiments, six wells/experiment and three locations/well). These data therefore indicate that different mutations differentially affect cell physiology.

DISCUSSION

In this study, we demonstrate different mutations in EGFR associate with different proteins, activate unique downstream signalling pathways (as shown by the induction of a unique set of genes and protein phosphorylation) and that cell lines expressing different EGFR mutation constructs display differences in physiology (proliferation and migration). Our data therefore demonstrate that different mutations have different functional consequences, which may provide an explanation for a tumour type specific mutation spectrum.

Our data are in line with other studies that highlighted differences between wildtype EGFR, EGFRvIII and/or EGFR p.L858R. For example, wildtype EGFR and EGFRvIII induce phosphorylation of different substrates, have differential activation of the JNK, STAT and MAPK signalling pathways, induce the expression of a unique set of genes and have differences in nuclear localisation [9–13,19]. Our data are also in line with a study showing that both wtEGFR and EGFRvIII interact with DNA–Protein Kinase (PRKDC) whereas EGFR p.L858R does not [19]: our biotin pulldown showed a ~two fold reduction in association with PRKDC of EGFRL858R-BG compared to both EGFRwt-BG and GFRvIII-BG (Supplementary Table 1). We did not observe differential association of EGFRwt-BG, EGFRvIII-BG and EGFRL858R-BG with CBL proteins, see [20]. However, binding to Cbl proteins occurs only after stimulation with EGF, whereas our cells were not EGF stimulated.

Apart from EGFR, a few proteins also show mutation specific binding partners and differential activation of downstream signalling pathways. Examples include TP53 (R273H and R267P) and PIK3CA [21–24].

Because tumours often remain dependent on their acquired genetic changes for growth, these changes are direct targets for treatment. However, when each mutation activates a unique set of downstream pathways, it is possible each mutation will require specific inhibition. Indeed, different mutations in EGFR show differential sensitivity towards

inhibitors: activating mutation in the kinase domain are associated with response to erlotinib and gefitinib whereas the EGFR p.A289D mutation is more sensitive to inhibition by lapatinib [7,25,26]. Moreover, kinase domain mutations do not occur in GBMs and inhibitors that act on these mutations (erlotinib and gefitinib) do not show clinical benefit in GBM patients eventhough EGFR is a driver in GBMs [27,28].

Our experiments demonstrate that a number of proteins differentially associate with EGFR constructs. It is interesting to note that mutations in DOCK4, UGGT1, MYCBP2 and SMTN have been found both in GBMs (2/283, 4/283, 1/283 and 1/283 respectively) and pulmonary adenocarcinomas (16/220, 7/220, 17/220 and 4/220). The first of these proteins that was further examined, DOCK4, associates with EGFRvIII-BG and, to a lesser extent, with EGFRwt-BG (but not with EGFRL858R-BG). DOCK4 is mutated in various tumors including bladder (~10%), colorectal (~10%) and lung (~7%). Two mutations in DOCK4 have thus far been identified in GBMs. DOCK4 is involved in cell migration through the activation of RAC1 [29,30]. Whether the difference in cell migration between EGFRwt-BG and EGFRvIII-BG is due to differential association with DOCK4 remains to be determined. DOCK4 also functions as a scaffold protein within the Wnt signaling pathway and is essential for activation of this pathway in vivo [18]. However, we did not find a mutation specific activation of the WNT pathway. A second differential binding protein, UGGT1, was found to predominantly associate with EGFRvIII-BG. UGGT1 plays a central role in the quality control of protein folding in the endoplasmic reticulum (glycosylated proteins) where it promotes substrate solubility [31]. It was recently demonstrated that the L858R mutation in EGFR reduces the disorganised conformation of the protein [32]. Because UGGT1 is involved in the quality control of protein folding, it is possible that the lack of association between UGGT1 and EGFRL858R-BG identified in our study may be a result of an altered (i.e. less disorganised) conformation.

Similar to UGGT1, MYCBP2 also showed preferential association with EGFRL858R-BG. MYCBP2 encodes an E3 ubiquitin ligase which mediates the ubiquitinylation and subsequent degradation of target proteins. The protein is involved in the regulation of the mTOR pathway: knockdown of MYCBP2 inhibits the mTOR pathway [33]. Finally, SMTN showed preferential association with EGFRL858R-BG. SMTN co-localised with a-actin and is involved in the contraction of smooth muscle cells [34]. Whether the differential association with specific EGFR mutations affects the mTOR pathway or actin dynamics in tumour cells remains to be determined.

EGFR is a member of the ERBB protein family, a family of proteins that plays a role in several cancer types [35]. The various ERBB family members can heterodimerise with

each other, and each heterodimer can activate different signal transduction pathways [35,36]. Although we demonstrate in this manuscript that different EGFR mutations activate unique molecular pathways, it remains to be determined whether the different mutations in EGFR also result in different heterodimerisation induced pathway activation. Of note, the various ERBB family members do not overtly show a tumor type specific mutation pattern [4,37].

Our results show that expression of EGFRvIII-BG or EGFRL858R-BG in HOG cells results in a decreased proliferation and migration, which may be counterintuitive for an oncogene. However, such reduced proliferation has been observed before in mutant melanoma cells where expression of EGFR confers a growth disadvantage that is further strengthened by the addition of EGF [38]. Perhaps this is caused when an oncogene (such as EGFR) is expressed in cells that have never been dependent on the oncogene (or the various mutations therein). A similar growth disadvantage (and altered migration pattern) was observed when expressing mutant (R132H) IDH1 into cell lines [39,40]. Interestingly, IDH1 also has a tumor-type specific mutation pattern [41,42].

In summary, our results indicate that there are distinct differences between different mutations in EGFR. Whether these different mutations also have different oncogenic properties remains to be determined. However, these functional differences can lead to the identification of mutation-specific EGFR inhibitors.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2015.02.006.

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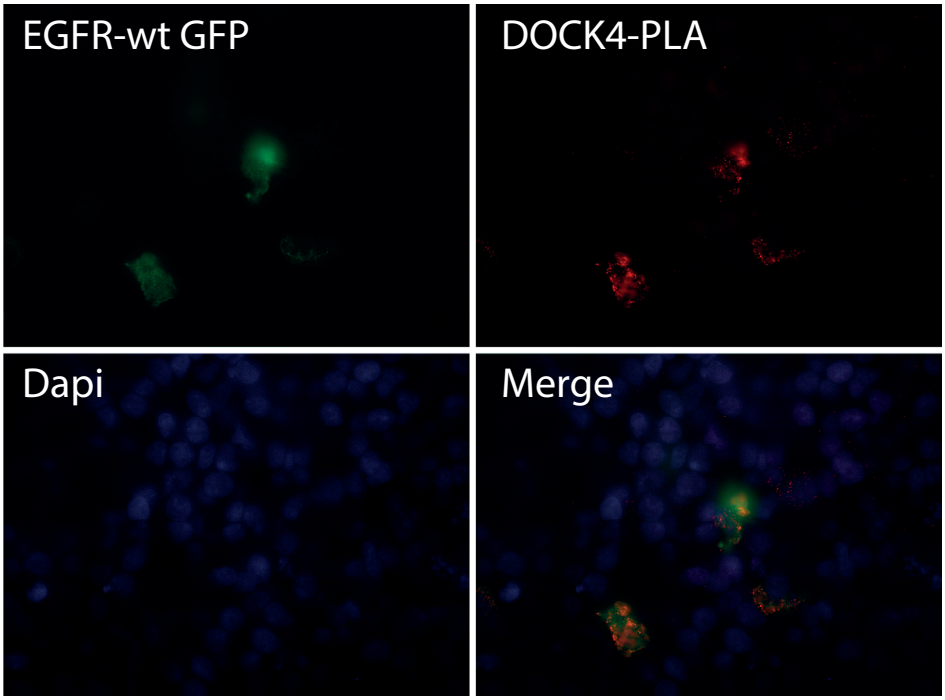
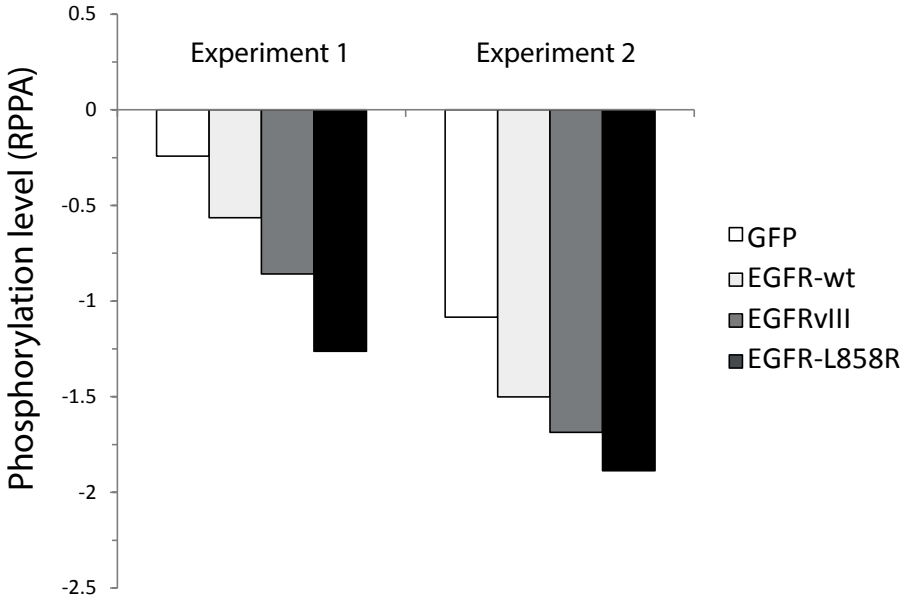


Supplementary Figure 1. A proximity ligation assay confirms that DOCK4 associated with EGFRwt-BG in **cells that** were serum starved overnight followed by EGF stimulation.



Supplementary Figure 2. Two independent RPPA experiments of Akt-pS473.