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Primary resistance to cetuximab in a panel of patient-derived tumour xenograft models: Activation of MET as one mechanism for drug resistance

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ABSTRACT

Cetuximab (Erbitux®) targets the epidermal growth factor receptor (EGFR) and is approved for treatment of colorectal and head and neck cancer. Despite wide expression of EGFR, only a subgroup of cancer patients responds to cetuximab therapy. In the present study we assessed the cetuximab response *in vivo* of 79 human patient-derived xenografts originating from five tumour histotypes. We analysed basic tumour characteristics including EGFR expression and activation, mutational status of KRAS, BRAF and NRAS, the expression of EGFR ligands and the activation of HER3 (ErbB3) and the hepatocyte growth factor receptor MET. Based on these results, a cetuximab response score including positive and negative factors affecting therapeutic response is proposed. Positive factors are high expression and activation of EGFR and its ligands epiregulin or amphiregulin, negative factors are markers for downstream pathway activation independent of EGFR. In cetuximab resistant NSCL adenocarcinoma LXFA 526 and LXFA 1647, overexpression due to gene amplification and strong activation of MET was identified. Knock-down of MET by siRNA in the corresponding cell lines showed that anchorage-independent growth and migration are dependent on MET. MET knock down sensitized LXFA 526L and LXFA 1647L to EGF. Combined treatments of a MET inhibitor and cetuximab were additive. Therefore, combination therapy of cetuximab and a MET inhibitor in selected lung cancer patients could be of high clinical significance.

1. Introduction

The human epidermal growth factor receptor (EGFR) is a well established drug target in colon, head and neck as well as non-small cell lung cancer (NSCLC). However, the population of patients responding to EGFR-targeted therapies is smaller than expected from the high proportion of patient tumours expressing EGFR, due to primary resistance.[1,2] In addition, most patients only respond transiently to therapy with EGFR-targeted drugs because of the development of acquired (secondary) resistance. Therefore, it is of importance to gain knowledge of the molecular mechanisms of drug resistance in order to identify patients with tumours that are fully addicted to an active EGFR, and also to guide the selection of combination treatments or alternative targeted therapeutics.

Cetuximab (Erbitux, C225) is a chimeric mouse-human monoclonal antibody of the IgG1 subclass that binds to the

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EGFR with ligand-competitive properties.[3] Besides inhibiting ligand binding, cetuximab disables the activated EGFR conformation required for receptor dimerization and phosphorylation,[4] inducing receptor downregulation. As a consequence, EGFR downstream signalling through the PI3K/Akt and Ras/Raf/Erk pathways is antagonized.[5,6] The roles of antibody-dependent cell-mediated cytotoxicity[7] and of autophagy[8] have not been extensively studied, but are likely to contribute to clinical efficacy.

Cetuximab is approved for the treatment of advanced colorectal cancer as well as for squamous cell carcinoma of the head and neck.[2,9] However, the response rate of cetuximab monotherapy with 10.8% in advanced colorectal cancer is low, and median time-to-progression is still only 1.5 months.[10] The low response rate is the result of primary (intrinsic) resistance, as described below, and includes mechanisms identified in acquired drug resistance. EGFR amplification and high expression of the ligands amphiregulin and epiregulin have been described for susceptibility to cetuximab.[2,11,12] Mutations in downstream components of EGFR signalling, most notably KRAS, NRAS and BRAF, are related to drug resistance. In metastatic colorectal cancer, KRAS mutations were found in about 33% of non-responding patients, but only in up to 10% of patients with disease control.[2] Also NRAS mutations in cetuximab treated colorectal cancer had lower response rates than wild-type tumours.[13] Activating BRAF mutations were identified in about 10% of patients and correlated to cetuximab resistance in some studies[11] but not others.[2] Similarly, phosphoinositol-3-kinase (PI3K) mutations possibly affect the cetuximab response, but conflicting data has been reported.[14] Acquired resistance to cetuximab has been experimentally addressed by continuous treatment of cell lines *in vitro*, which attained resistance by (i) upregulation of alternative growth factor receptors, namely HER2 (ErbB2) and HER3 (ErbB3), (ii) MET activation,[15] (iii) obstruction of EGFR interaction with c-Cbl, leading to failure of EGFR downregulation[16] and finally (iv) high activity of Src family kinases.[17]

The EGFR tyrosine kinase inhibitors (TKI) gefitinib (Iressa®) and erlotinib (Tarceva®) are approved for the treatment of advanced NSCLC and erlotinib for pancreatic cancer. Acquired resistance in patients treated with these drugs is mainly due to mutations in the kinase domain.[18,19] Primary resistance mechanisms by activation of alternative receptors have been described. The HER2 variant $HER2^{YVMA}$ is expressed in 4% of NSCLC[20] and confers resistance by ligand-independent EGFR activation.[21] Expression of the insulin-like growth factor-1 receptor (IGF-1R) has been implicated in weakened response of NSCLC cell lines to TKIs, but not to cetuximab.[22,23] A combination of gefitinib with an IGF-1R targeted antibody prevented recurrence of the A431 xenograft.[24]

MET contributes to primary and acquired resistance to TKIs. MET is the receptor for hepatocyte growth factor (HGF, also called scatter factor) and its constitutive activation in cancer leads to invasive growth and metastasis.[25] MET amplification and signalling via HER3 have been found in preclinical models of acquired resistance and in NSCLC patients.[26] High HGF expression has been described in 40% of patients progressing under gefitinib therapy, but not in the original lung tumours.[27] Further, MET was highly expressed in an EGFR/HER2 inhibitor resistant NSCLC cell line and interacted with EGFR and HER2.[28] In accordance with this, 6% of NSCLC patients had high levels of phosphorylated MET, associated with resistance to gefitinib.[29] Nevertheless, a role of MET in primary resistance to cetuximab has not been established so far.

In the present study we investigated the response towards cetuximab of 79 different patient-derived tumour xenografts, generated at Oncotest through implantation of primary patient material and shown to be of high clinical relevance.[30] [XF, Xenograft Freiburg (cancer histotypes: CXF, colon, GXF, gastric, HNXF, head and neck, LXF, lung (LXFA, adenocarcinoma, LXFE, epidermoid, LXFL, large cell), MAXF, mammary).] In addition to cetuximab activity *in vivo*, KRAS, BRAF and NRAS mutational status, EGFR ligand expression and pathway activation, as well as MET, HER2 and HER3 activation were investigated. In a representative subgroup changes in EGFR and downstream pathway activation upon cetuximab treatment were analysed. The cetuximab-resistant lung adenocarcinoma LXFA 526 and LXFA 1647 were studied in more detail. Both tumours expressed very high levels of activated MET concurrent with cetuximab resistance..

2. Materials and methods

2.1. *Reagents and antibodies*

Erbitux was from Merck-Serono, EGF from R&D systems, 5FU from Sigma, PF-04217903 from Selleck Chemicals LLC, XTT assay from Roche, bead suspension assays of total and phospho Erk1/2, Akt, and phospho EGFR, p70S6K from Bio-Rad and total EGFR, p70S6K from Millipore. Antibodies were: MET (25H5), pMET (3D7) (Cell Signalling Technology), EGFR (Millipore), β-actin and GAPDH (Abcam), mouse IgG (Santa Cruz Biotechnology), and phospho ErbB3 ELISA was from R&D.

2.2. *Cell lines and xenograft models and cetuximab treatment*

All xenografts (except HT29 (NCI), DiFi provided by Dr. Trusolino[31], PC14 (ECACC), MX1 (NCI) were established at Oncotest GmbH from primary patient material after the informed consent of the patients. Xenografts were subcutaneously grown and randomized after reaching tumour volumes of about 100 mm^3. Mice were treated with 30 mg/kg cetuximab intraperitoneally (i.p.) on days 0 (randomization), 7 and 14 and the vehicle control was PBS. Tumour sizes were measured twice weekly until study day 28 and from the median relative tumour volumes the treatment versus control (T/C [%]) values were calculated. Optimal T/C values were used to assess cetuximab response. All studies were done in agreement with German animal welfare acts.

Cell lines LXFA 526L and LXFA 1647L were established from the xenografts and cultivated under standard conditions in RPMI ready (PAA). Authenticity of cell lines was confirmed by STR analysis.

2.3. Bio-Plex assays, ELISA and Western blot

Tumours for native protein lysates were explanted and snap frozen 24 h, 48 h or 72 h after one dose of cetuximab or vehicle. Tumour numbers: $n = 1$; except for GXF 251 and CXF 742 $n = 3$ control, 24 h and 72 h, $n = 0$ 48 h. Three parts of the tumour xenograft were homogenized by Tissue Lyser (Qiagen) in cell lysis buffer (Bio-Rad) and lysates cleared and concentration measured (Protein Assay, Bio-Rad).

Bio-Plex assays and ELISA assays were according to the manufacturers' protocols. The Oncotest tumour lysate pool containing a mixture of 106 different patient-derived tumour lysates was used as a normalization control to minimize plate-to-plate variability. Median Fluorescence Intensity (MFI) or Optical Density (OD) of the Oncotest lysate pool was defined as 1 and all samples were normalized against this to calculate normalized Median Fluorescence Intensity (nMFI) or normalized Optical Density (nOD) values. For immunoblotting protein lysates were boiled with Laemmli buffer (Bio-Rad), separated by electrophoresis, blotted on PVDF membrane, blocked, incubated overnight with primary antibody, 1 h with secondary antibody, and developed on Hyperfilm ECL (GE Healthcare).

2.4. Immunoprecipitation

For immunoprecipitation cell lysates were made in buffer 150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 5 mM EDTA, inhibitors Na_3VO_4, NaF, PMSF, Protease Mix (Roche) and the detergent digitonin (Sigma) 1:15. 1 ml cleared lysates at 1 μg/μl in buffer with 1:150 digitonin were incubated with mouse IgG or MET antibody at 4 °C, for further 2 h with protein A/G Sepharose (GE Healthcare), washed three times with buffer, and boiled with 50 μl Laemmli buffer.

2.5. Cell transfection, clonogenic and wound healing (scratch) assays

LXFA 526L and LXFL 1647L cells were transfected (Dharmafect) with scrambled or MET SmartPool siRNA at 50nM (Dharmacon). After 24 h 10 ng/ml EGF was added. After 48 h cells were harvested, counted and plated in 0.4% agarose (LXFA 526L 7×10^3, LXFA 1647L 5×10^3 cells). After 4 h 0.66 μM cetuximab, 10 ng/ml EGF was added. For compound treatment LXFA 526L and LXFL 1647L cells were plated in 0.4% agarose and treatment began after 24 h. On day 4–5 colonies counted as described[32]. For the wound healing assay, two scratches in the cell monolayer of siRNA-transfected LXFA 526L were made with a fine pipette tip, photographs after 72 h.

2.6. DNA Extraction, PCR and Sequencing

DNA was isolated from xenografts (DNeasy, Qiagen) and KRAS exons 2, 3 (NM_004985), BRAF exons 11, 15 (NM_004333) and NRAS exons 1, 2 (NM_002524) amplified by PCR. Sequencing with BigDye® Terminator Cycle Sequencing Kit and ABI 3730 XL Genetic Analyzer (Applied Biosystems).

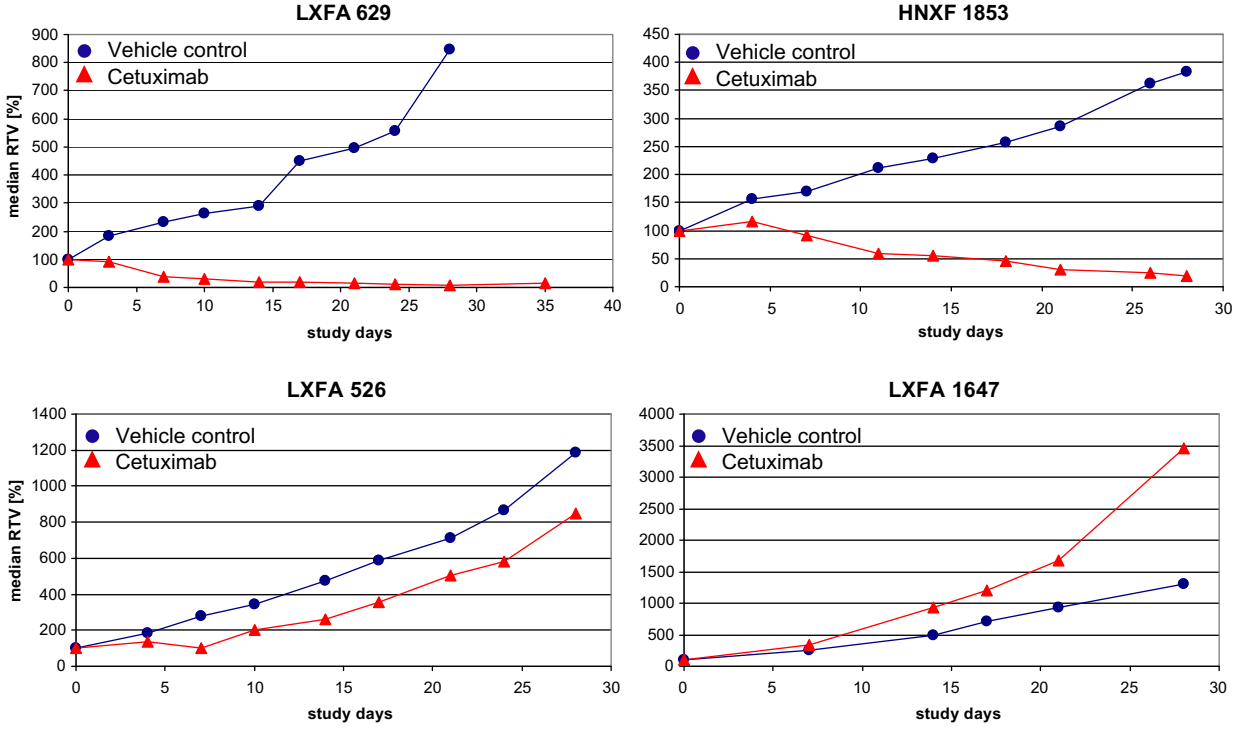


Fig. 1 – Cetuximab activity. Cetuximab sensitivity of patient-derived xenografts was determined by treating tumour-bearing nude mice with 30 mg/kg cetuximab or the vehicle control PBS i.p. once weekly for 3 weeks. Relative tumour volumes (RTV) are plotted against the time after first treatment (day 0). Examples for growth curves of tumours with (▲) and without (●) cetuximab treatment for sensitive models LXFA 629 and HNXF 1853, and for resistant models LXFA 526 and LXFA 1647.

Primer:		
KRAS exon 2	GATACACGTCTGC AGTCAACTG	GGTCCTGCACCA GTAATATGC
KRAS exon 3	GGTGCACTGTAA TAATCCAGACT	CATGGCATTAGCAAA GACTCA
NRAS exon 1	GATGTGGCTCG CCAATTAAC	GGTAAAGATGATCC GACAAGTG
NRAS exon 2	GCAATTTGAGGG ACAAACCA	TGGTAACCTCATTT CCCCATA
BRAF exon 11	AGAATTTTTCTTAA GGGGATCTCTTC	TTGAGGACTAGTT AACCTGGAGGA
BRAF exon 15	TTAGGAAAGCATCT CACCTCATC	CACTGATTTTTGTG AATACTGGGA

2.7. *Affymetrix HG U133 Plus 2.0 gene expression analysis*

RNA from xenografts was analysed on Affymetrix HG U133 plus 2.0 arrays. Expression values were normalized according to the median (=50 percentile) expression value of all genes on the array (=1). The Affymetrix probe sets were 205767_at for epiregulin (EREG) and 205239_at for amphiregulin (AREG).

3. Results

3.1. *Anti-tumour activity profile of cetuximab in a panel of patient-derived tumour xenografts*

Anti-tumour activity of cetuximab was tested in a panel of 79 patient-derived xenografts at a dose of 30 mg/kg, which is equivalent to the clinical maintenance dose in humans, given i.p. once weekly for 3 weeks (qd7x3). Growth curves of four representative experiments are shown in Fig. 1. The NSCLC adenocarcinoma LXFA 629 and the head and neck cancer HNXF 1853 are examples of cetuximab sensitive and the NSCLC adenocarcinoma LXFA 526 and LXFA 1647 for resistant tumours. The cetuximab response of individual tumour models varied between optimal treatment/control (T/C) values of <1% and 100%. Out of the 79 patient-derived tumour xenografts tested, 16 were rated as responsive (optimal

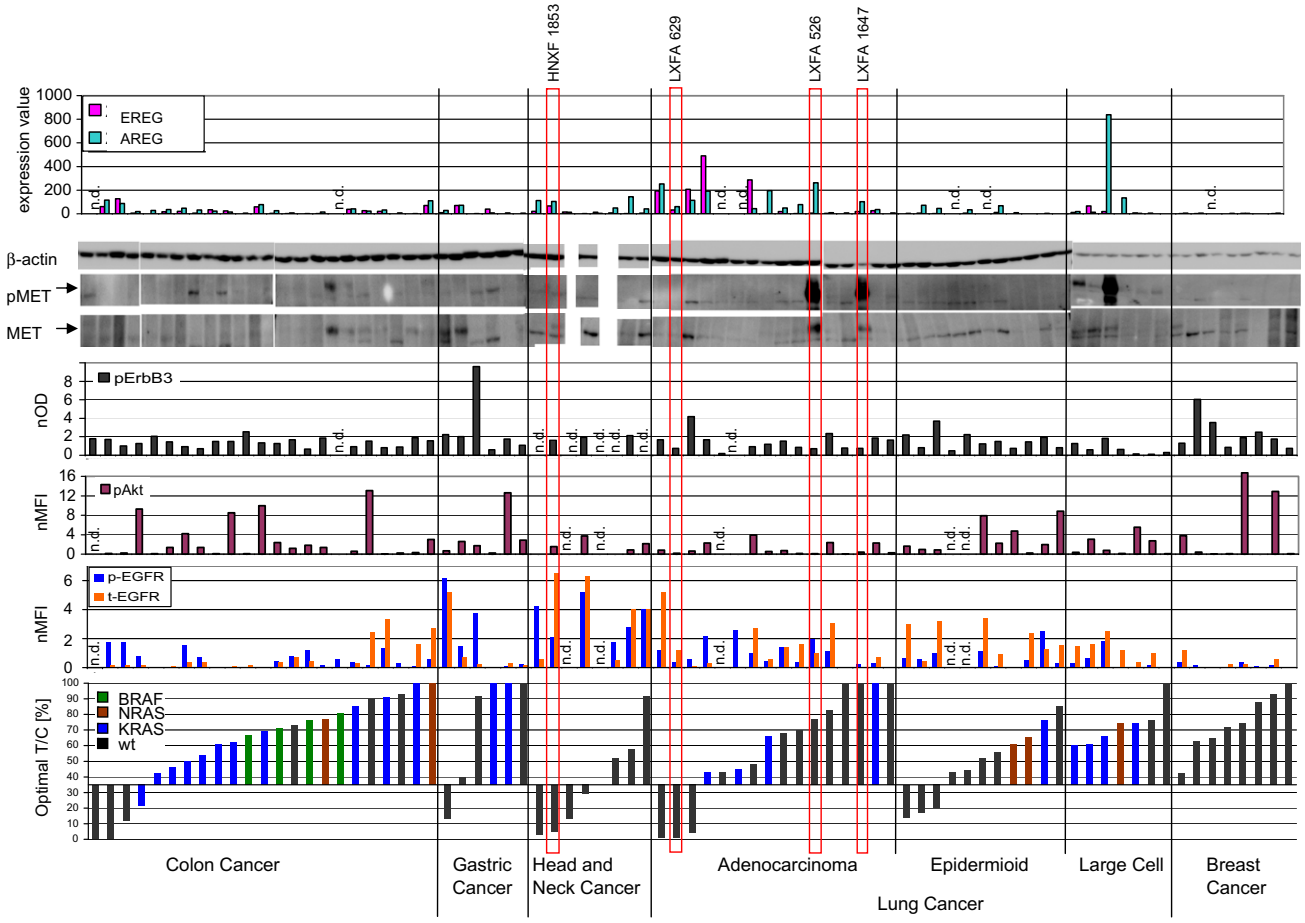


Fig. 2 – Cetuximab activity profile in 79 patient-derived tumour models. (From bottom to top) Cetuximab sensitivity of patient-derived xenografts was determined by treating tumour-bearing nude mice i.p. once weekly for 3 weeks. The optimal T/C values are plotted in a waterfall plot with the cutoff for sensitivity at 35%. Mutations in BRAF, KRAS and NRAS are shown by colour coding. BRAF mutation – green, KRAS mutation – blue, NRAS mutation – brown, wild type – black. Phosphorylated EGFR, total EGFR and phosphorylated Akt were measured by Bio-Plex in tumour lysates and normalized to the Oncotest lysate pool (for Oncotest lysate pool nMFI = 1). Phosphorylated ErbB3 (HER3) was analysed by ELISA and the OD also normalized to the Oncotest lysate pool (nOD). MET and phosphorylated MET and the loading control β-actin were analysed by Western blot. Epiregulin (EREG_205767_at) and amphiregulin (AREG_202539_at) were measured by Affymetrix.

T/C ⩽ 35% Fig. 2, lowest panel) and 10 displayed tumour regression (relative tumour volume < 70% at least at one time point). Responsiveness was found in colon carcinoma (4/23, 17%), head and neck cancer (5/8, 63%), gastric cancer (1/6, 17%), NSCLC of adeno histology (3/16, 19%) and epidermal (3/11, 27%) histologies, but not in NSCLC of large cell cancer (0/7, 0%) or breast cancer (0/8, 0%).

3.2. *Molecular characteristics of patient-derived tumour xenografts*

For the molecular characterization factors known to be involved in the response to EGFR targeting agents were analysed (Fig. 2): Mutation of KRAS, BRAF and NRAS, expression and activation of the EGFR, epiregulin and amphiregulin ligand expression, MET expression and activation and HER3 activation. Akt phosphorylation was measured to show downstream activation, which could be dependent or independent from EGFR.

Colon cancer xenografts showed a high proportion of KRAS, BRAF and NRAS mutations. Here 84% (16/19) of the resistant tumours had an activating mutation in one of the three genes; most were KRAS mutations (11/19). 50% (3/6) of tumours without any of these mutations responded to cetuximab, whereas of the mutated tumours only one out of 17 showed response. This demonstrates a pivotal role of the analysis of KRAS, BRAF and NRAS in the selection of colon carcinoma which may respond to cetuximab therapy.

Of six gastric carcinoma tested, one had very high levels of EGFR expression and activation, responding to cetuximab with tumour regression. Of the four tumours with optimal T/C values > 90%, one had amplified HER2 (data not shown), which may lead to EGFR independence, and two were mutated in KRAS.

Of the eight head and neck cancers analysed, 63% (5/8) were responsive. This is a higher proportion than in any other tumour histotype studied here. However, the reasons for lack of response of HNXF 1838, HNXF 536 and HNXF 700 could not be traced to lack of EGFR activation, nor to the overexpression of alternative receptors HER3 or MET, nor to mutations in KRAS, BRAF or NRAS.

The lung cancers of different histotypes were analysed separately. Adenocarcinoma of the lung were found to be most responsive when (i) EGFR was highly expressed, as well as the ligands epiregulin and amphiregulin, (ii) no Ras/Raf pathway mutation was detected and (iii) no high expression and activation of MET. KRAS mutations were found in 31% (4/13) of resistant but not in sensitive NSCL adenocarcinoma. All KRAS mutant tumours were cetuximab resistant. Two out of 13 tumours, namely LXFA 526 and LXFA 1647, had very high levels of MET expression and activation. They were studied to test the significance of their MET expression for cetuximab resistance (see below). In NSCL epidermoid carcinoma 38% (3/8) non-responsive tumours had an activating mutation in KRAS (1/8) or NRAS (2/8). In NSCL large cell carcinoma 57% (5/7) had such a Ras/Raf pathway mutation (KRAS 4/7, NRAS 1/7). Two tumours had high phosphorylated MET expression, and both of these were also KRAS mutant, combining two characteristics unfavourable to cetuximab response.

None of the mammary carcinoma responded to cetuximab. Only one of the breast tumours had high expression of EGFR, and also showed the most favourable response with an optimal T/C of 42%.

3.3. *Proposing a cetuximab response score (CRS)*

Individual tumour characteristics have been useful to classify certain tumours as cetuximab responsive or non-responsive. For example KRAS mutations are unfavourable to cetuximab response. However, among KRAS wild type tumours many do not respond to cetuximab. Therefore, we combined analysis of various molecular characteristics to a 'cetuximab response score' (CRS).

As EGFR is the target of cetuximab it plays a central role in analysis of response. EGFR and phosphorylated EGFR were measured by Bio-Plex and correlated with the optimal T/C. Both correlated with response (phosphorylated EGFR $p = 0.001$, $R^2 = 0.141$, EGFR $p = 0.008$, $R^2 = 0.095$, Fig. 3A). Classification of the cetuximab resistant and sensitive tumours solely by the expression and activation of EGFR is correct for most tumours with an nMFI > 4 and where no receptor activity was measured. Combined with further molecular characteristics, classification becomes more accurate. High expression

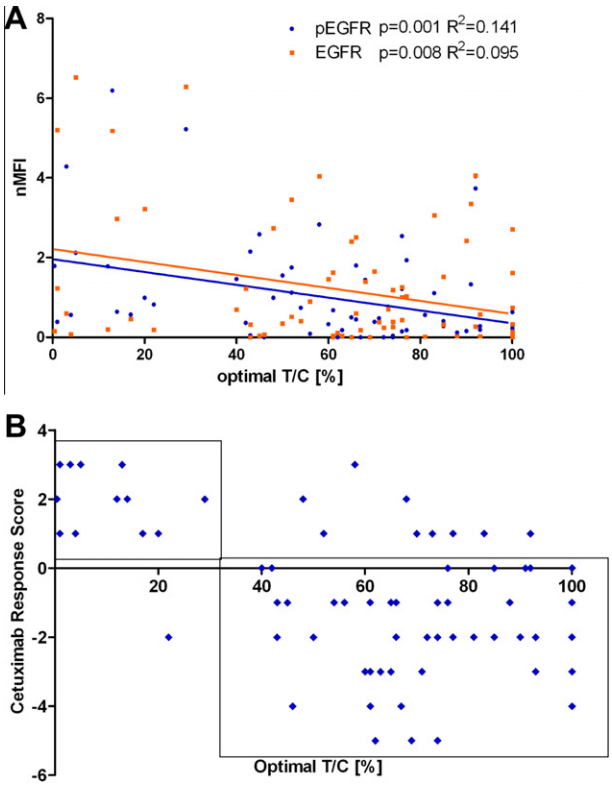
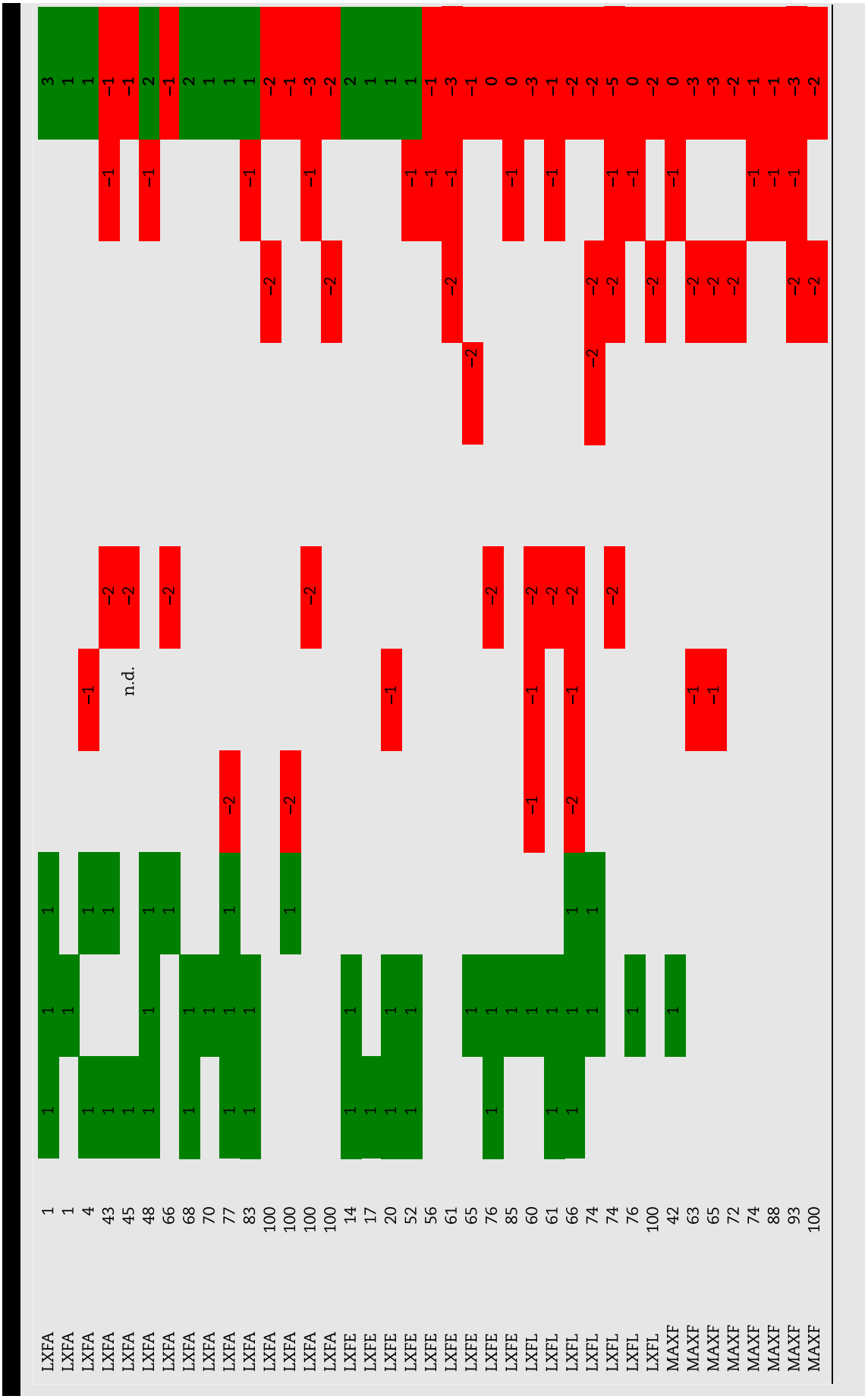


Fig. 3 – Correlation with cetuximab response. (A) For 73 tumours EGFR expression and phosphorylation was measured by Bio-Plex assays and normalized to the Oncotest lysate pool. The nMFI values are plotted against the optimal T/C values of the tumours' response to cetuximab. (B) The cetuximab response score (CRS) calculated as in Table 1 was plotted against the optimal T/C values. The tumour models in the boxed areas were correctly classified.

Table 1 – Cetuximab Response Score (CRS). The table shows the calculation of the CRS for 73 tumours. The factors counted as positive points are in green, those counted as negative points in red. White fields indicate that a tumour did not exhibit this criterion, n.d. indicates that a value was not determined in a certain tumour. The score is coloured green, when 1 and above and red when 0 or below.

	T/C [%]	High phospho EGFR	High EGFR	High Amphiregulin or Epiregulin	High phospho MET	High phospho ErbB3	KRAS mutation	BRAF mutation	NRAS mutation	No phospho EGFR detectable	>2x pool phospho Akt	**Cetuximab Response Score**
CXF	0.4	1		1								2
CXF	12	1		1								2
CXF	22	1					−2				−1	−2
CXF	43						−2					−2
CXF	46						−2			−2		−4
CXF	50	1					−2				−1	−2
CXF	54	1					−2					−1
CXF	61						−2			−2		−4
CXF	62						−2			−2	−1	−5
CXF	67							−2		−2		−4
CXF	69						−2			−2	−1	−5
CXF	71							−2			−1	−3
CXF	73	1										1
CXF	76	1						−2				−1
CXF	77									−2		−2
CXF	81	1			−1	n.d.		−2				−2
CXF	85						−2					−2
CXF	90		1							−2	−1	−2
CXF	91	1	1				−2					0
CXF	93								−2			−2
CXF	100		1				−2					−1
CXF	100	1	1	1					−2		−1	0
GXF	13	2	1									3
GXF	40	1									−1	0
GXF	92	1				−1						0
GXF	100						−2			−2		−4
GXF	100						−2				−1	−3
GXF	100										−1	−1
HNXF	3	2		1		n.d.						3
HNXF	5	1	1	1								3
HNXF	29	2	1								−1	2
HNXF	52	1				n.d.						1
HNXF	58	1	1	1								3
HNXF	92	1	1			n.d.					−1	1


LXFA 1
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LXFL 76
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MAXF 42
MAXF 63
MAXF 65
MAXF 72
MAXF 74
MAXF 88
MAXF 93
MAXF 100
n.d.

and phosphorylation of EGFR as well as high ligand (amphiregulin or epiregulin) expression were counted in favour of response. High activity of MET and HER3, mutations in the Ras/Raf pathway, EGFR phosphorylation below the level of detection and a very high amount of phosphorylated Akt were counted as negative predictors. Akt could be highly phosphorylated due to activation of the EGFR pathway or alternate upstream signalling, but was counted as a negative point even when EGFR was highly activated. KRAS, BRAF and NRAS mutations and lack of phosphorylated EGFR were weighted double, as was EGFR phosphorylation above 4 nMFI. A CRS of 1 and higher defined responsive tumours, a score of 0 and lower indicated resistant tumours. This classification was correct for 86% (63/73) of tumours (Table 1 and Fig. 3B). The tumours incorrectly classified were mostly false positive (12.3%, 9/73) and one (1.3%, 1/73) tumour was falsely classified as non-responsive. In the different tumour types 90% (18/20) of colon carcinoma, 100% of gastric carcinoma, 50% (3/6) of

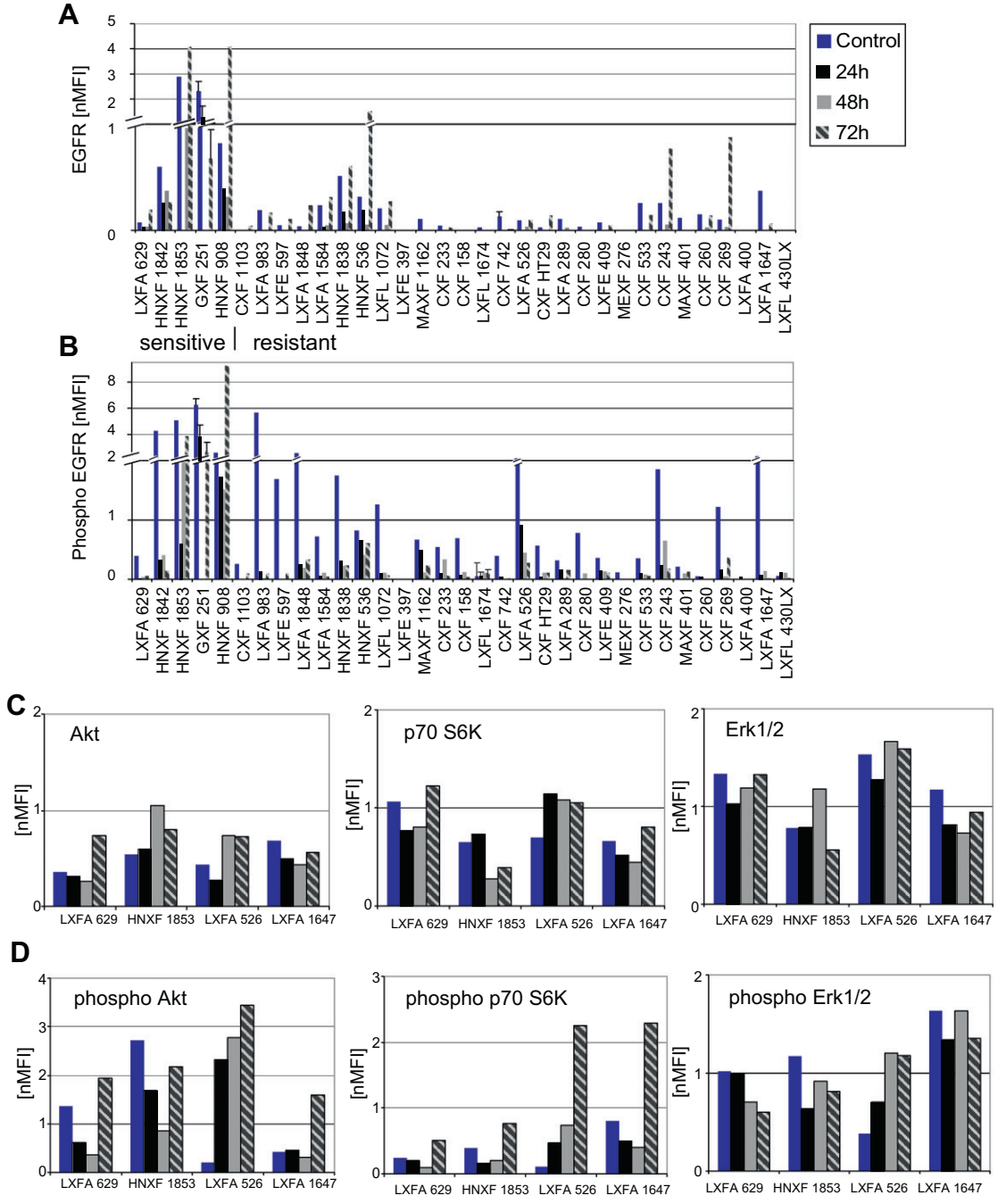

Fig. 4 – Effects of cetuximab on EGFR and downstream signalling components. Tumour-bearing nude mice were treated once with 30 mg/kg cetuximab, tumours were harvested after 24 h, 48 h, 72 h and native protein lysates analysed by Bio-Plex assays. (A) total EGFR expression, (B) phosphorylated EGFR, (C) selected examples analysed for total and phosphorylated (D) protein levels of Akt, Erk1/2 and p70 S6K.

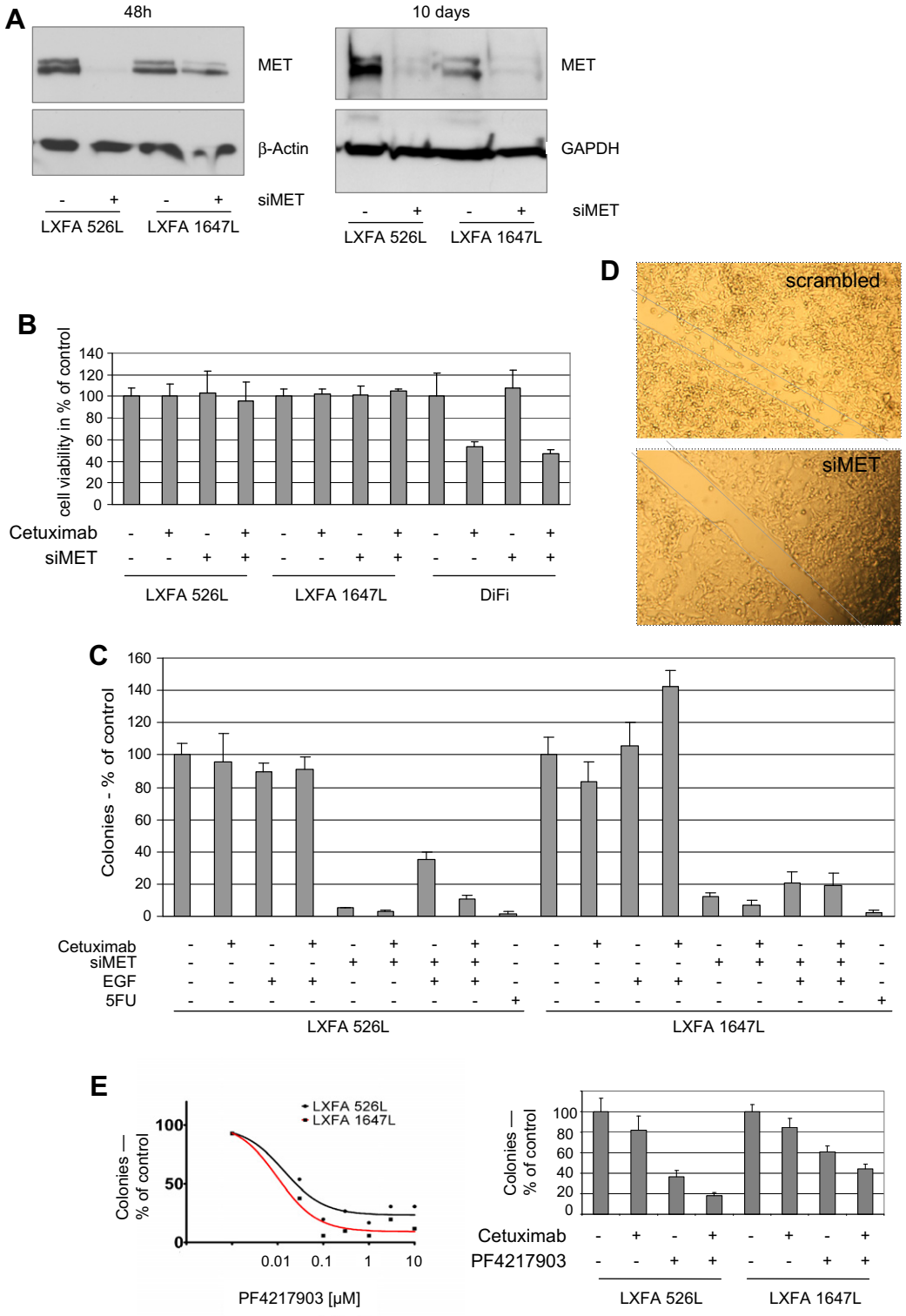

Fig. 5 – Functional consequences of MET knock-down. (A) Western blot analysis of MET knock-down by siRNA in LXFA 526L and LXFA 1647L cells. (B) Proliferation/viability of cells grown in monolayer culture measured by the XTT assay (siRNA for MET or scrambled control; treatment with 5 μM cetuximab for 4 d). (C) Clonogenicity of LXFA 526L and LXFA 1647L was evaluated under different conditions: siRNA for MET or scrambled control; treatment with 10 ng/ml EGF overnight before seeding into soft agar (n = 3); treatment with 0.66 μM cetuximab and 10 ng/ml EGF as indicated. 5-Fluorouracil (5-FU) at 7.7 mM served as a positive control. (D) Motility of LXFA 526L cells after knock-down of MET analysed by the wound healing assay. (E) Clonogenicity of LXFA 526L and LXFA 1647L with titration of the MET inhibitor PF-04217903 and combination of PF-04217903 at 0.1 μM with 0.66 μM cetuximab.

head and neck cancer, 81% (25/31) of NSCLC and 100% (8/8) of mammary carcinomas were correctly classified. The numbers of head and neck cancer analysed are low, yet a cautious suggestion can be made, that resistance to cetuximab treatment in head and neck cancer is not defined by alternate receptor activation or Ras/Raf pathway mutation.

The power of correct classification of the CRS was better for colon cancer than analysis of Ras/Raf mutations only (90% versus 82%), for NSCLC (81% versus 52%) and gastric cancer (100% versus 50%).

3.4. *Modulation of target and downstream signalling by cetuximab in vivo*

Cetuximab competes with ligand binding and mediates EGFR internalization and degradation. We therefore investigated the effects of cetuximab on EGFR expression and phosphorylation in 33 tumour xenografts *ex vivo* (Fig. 4A/B). For this proteomic analysis, animals where treated with a single dose of 30 mg/kg cetuximab before lysis of tumours 24 h, 48 h and 72 h later. About 85% of all tumours (28/33) had measurable basal EGFR expression. All tumours with detectable EGFR expression exhibited an at least twofold reduction 24 h or 48 h after treatment. A potential resistance mechanism through lack of EGFR degradation due to diminished Cbl binding[15] was not present in any of the tumours examined. Interestingly, upregulation of the EGFR after 72 h was evident in selected tumours like CXF 234 and HNXF 908, classified as therapy resistant and sensitive, respectively. Activation of EGFR was high (phospho EGFR nMFI > 1) in 36% of the tumour models analysed (12/33; Fig. 4B). In most tumours with detectable phosphorylated EGFR, cetuximab caused a strong reduction (26/28, Fig. 4B) which was not correlated to response.

Next, we analysed downstream signalling components of the EGFR pathway, namely Akt, p70 S6K and Erk1/2, in the cetuximab sensitive tumours LXFA 629 and HNXF 1853 and the resistant tumours LXFA 526 and LXFA 1647. In general, the expression of Erk1/2, Akt and p70 S6K proteins was not much affected by cetuximab treatment (Fig. 4C). Regarding phosphorylated, active kinases a more complex picture emerged (Fig. 4D). In LXFA 629 and HNXF 1853 Akt activity was reduced after 24 h and 48 h (Fig. 4D). p70 S6K and

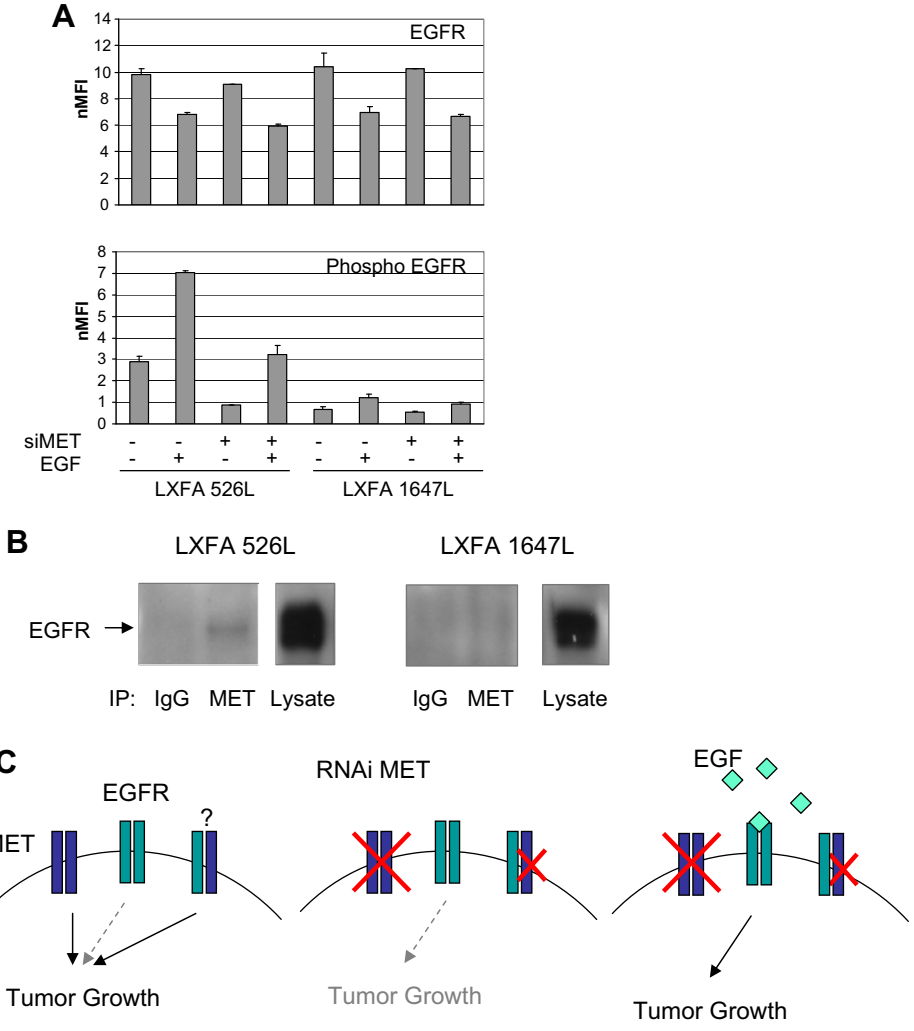

Fig. 6 – Interaction of MET and EGFR. (A) Total and phosphorylated EGFR were measured by Bio-Plex assays in lysates of LXFA 526L and LXFA 1647L cells treated as described in Fig. 5C. (B) Co-immunoprecipitation of MET and EGFR in LXFA 526L and LXFA 1647L cell lysates. (C) The cartoon depicts a hypothesis for the effects of MET knockdown on LXFA 526L.

Erk1/2 phosphorylation were only slightly reduced. Surprisingly, the NSCL adenocarcinoma LXFA 526 markedly upregulated Akt, p70 S6K as well as Erk1/2 phosphorylation at all time points measured (Fig. 4D). In the other tumours, Akt and p70 S6K activity was elevated 72 h after treatment, regardless of response to therapy. Erk1/2 activation was not strongly modified. This implies a feedback mechanism that affects the PI3K/Akt, but not the Ras/Raf/Erk pathway.

3.5. *Constitutive MET activation as a mechanism for primary cetuximab resistance*

High activation of MET was found in four NSCLC tumours of the cetuximab response panel. LXFA 526 and LXFA 1647 were wild type for KRAS and chosen for evaluation of a role of MET in primary cetuximab resistance.

To examine the function of MET in LXFA 526 and LXFA 1647 NSCL adenocarcinoma, MET was knocked-down in the corresponding tumour cell lines LXFA 526L and LXFA 1647L by siRNA. The knock-down was evident for at least 10 d on the protein level (Fig. 5A). Functional consequences of MET knock-down were studied in a monolayer proliferation assay, in a clonogenic assay to assess growth in a three-dimensional matrix and by a wound healing (scratch) assay. Cell viability was not affected by the knock-down of MET, cetuximab treatment or the combination of both (Fig. 5B). The highly cetuximab-sensitive adenomatosis polyposis coli cell line DiFi was included as a positive control. In contrast to cell proliferation, LXFA 526L and LXFA 1647L required MET for clonogenic growth in soft agar (Fig. 5C), so did LXFA 526L for spreading (Fig. 5D). This result is consistent with the function of MET in cell motility, spreading and anchorage-independent growth.[25] Cetuximab had no effect on clonogenic growth of LXFA 526L cells, nor had EGF. However, after knock-down of MET, EGF-induced clonogenic growth was attained, and partly suppressed by cetuximab (Fig. 5C). LXFA 1647L cells displayed a slightly different behaviour: although the extent of the stimulatory effect of EGF on colony formation in cells with MET knock-down was variable between experiments, EGF responsiveness was reproducible. In each case, cetuximab did not significantly reduce the EGF-induced colony formation after MET knock-down.

Clonogenic growth of LXFA 526L and LXFA 1647L was also antagonized by the MET inhibitor PF-04217903, with an IC_{50} of 16 nM and 13 nM, respectively. An additive effect of the MET inhibitor with cetuximab was found for both cell lines and was more pronounced for LXFA 526L (Fig. 5E).

These results show that MET is highly important for the malignancy of selected lung adenocarcinomas, and that the combination of MET and EGFR inhibition is additive.

3.6. *Effect of MET knockdown on EGFR activation*

Crosstalk of EGFR and MET has been published for the NSCLC cell lines H441, HCC827 or H355.[28,33] We analysed LXFA 526L and LXFA 1647L cell lines for a functional interaction between MET and EGFR. MET knock-down in LXFA 526L cells reduced basal and EGF-stimulated EGFR phosphorylation (Fig. 6A). EGFR downregulation 24 h after EGF stimulation is probably due to ligand-induced receptor degradation.[34] In LXFA 1647L cells, the EGFR activity in general was lower, and a slight reduction of phosphorylated EGFR by MET knock-down was observed. In co-immunoprecipitation studies, a physical interaction of endogenous MET and EGFR in LXFA 526L cells was shown (Fig. 6B). We conclude that MET and EGFR are linked to cetuximab resistance in LXFA 526 and to a lesser degree in LXFA 1647.

4. Discussion

The clinical use of cetuximab and other targeted cancer therapeutics is ultimately linked to an understanding of the factors, which render tumours susceptible or resistant to treatment. In the present study we determined the response to cetuximab therapy in 79 patient-derived tumour xenografts. The overall rate of tumour response was 13%.

The prediction of cetuximab response remains elusive. Activating mutations of KRAS, BRAF and NRAS are common in human malignancies, like colorectal and NSCL cancer.[35,36] In the treatment of metastatic colorectal cancer, distinguishing patients who may benefit from treatment by KRAS mutation status is a major achievement.[37,38] The combination of KRAS mutations with PI3K, PTEN and BRAF abnormalities further improves the prediction.[39] The present study supports the finding that mutated KRAS is a negative prognostic factor, and that its predictive power can be enhanced by analysis of additional molecular characteristics. In the present study we had the opportunity to compare tumours of different histotypes on a molecular level in their chemonaive state. We defined a 'cetuximab response score' (CRS) by combining this information. The score was able to distinguish sensitive from resistant tumours in 86% of cases. Highly sensitive tumours exhibited positive criteria, namely high phosphorylation and expression of EGFR, high expression of amphiregulin or epiregulin and the absence of negative predictors. In therapy-resistant tumours, positive response criteria were overruled by negative predictors, KRAS, NRAS and BRAF mutations, no detection of activated EGFR, activation of alternative receptors and high activation of Akt, which lead to EGFR independency. PIK3CA mutations were also analysed and did not correlate to cetuximab response (data not shown), nor did EGFR mutations (Suppl. Table 1). The exact substitution of an activating mutation of KRAS result in a significant difference in response[40] (Suppl. Table 2).

The CRS demonstrates that a tumour's response to therapy is determined by its individual pattern of markers. Prediction of response by biomarker analysis is more likely to succeed, if it takes a number of tumour characteristics into account.

Beside basal pathway activation in tumours *in vivo*, we were interested in understanding pathway alterations induced by cetuximab treatment. EGFR expression and phosphorylation was diminished in sensitive as well as resistant tumours 24 h and 48 h after treatment. We found that many tumours responded to cetuximab with a late activation of signalling components, most notably p70 S6K. This points towards a counter-regulation through the PKB/Akt signalling pathway and a rational for combination therapies. Everolimus, an inhibitor of mTOR, which is upstream of p70 S6K, has been shown to synergize with the EGFR TKI gefitinib.[41]

In depth studies are needed to understand this upregulation of key signalling molecules in response to EGFR antagonism at late time points. One may speculate that EGFR signalling, independent from its necessity for tumour growth, activates negative feedback loops which result in Akt activation upon EGFR inhibition.

Independence from EGFR can be caused by activation of alternative receptors, e.g. HER3 or MET. HER3 phosphorylation was particularly high, in two mammary tumours which also have HER2 amplification. One mechanism of HER3 signalling is through heterodimerization with HER2,[42] which is possibly the case here. Overexpression and constitutive activation of MET was identified in the resistant NSCL tumours LXFA 526 and LXFA 1647. In the respective xenograft-derived cell lines LXFA 526L and LXFA 1647, MET was essential for anchorage-independent growth and additional stimulation by EGF did not enhance colony formation. In contrast, MET was dispensable for proliferation and survival of either cell line in monolayer cultures. Dependence of proliferation in monolayer cultures on MET is rare and only found in a few, exceptional cell lines like the gastric carcinoma MKN45 (data not shown).

Co-expression of EGFR and MET and interaction of their signalling pathways has strong implications for cancer therapy. In the *in vitro* clonogenic assay with LXFA 526L and LXFA 1647L, MET knock-down restored EGF responsiveness and MET inhibition with PF-04217903 and EGFR inhibition by cetuximab showed additive effects in the same assay. The impact of a given signalling pathway is determined (i) by the level of addiction to a particular oncoprotein like MET or EGFR and (ii) by the availability of growth factors in the tumour microenvironment. Combination of inhibitors of MET and EGFR has been successful in some studies including recent clinical trials.[43–47] Crosstalk between MET and EGFR family receptors has been shown to affect sensitivity to TKIs in *in vitro* studies and in cancer patients.[26,28]. In colorectal cancer MET amplification by FISH was not shown to produce resistance to cetuximab.[48] However, MET activation was not shown, and may be a mechanism of resistance to EGFR targeting in NSCLC rather than colorectal cancer. In recent studies, interaction of MET and EGFR have been shown, which may contribute to EGFR activation.[26,28] We could show a physical interaction between EGFR and MET in LXFA 526L, as well as functional interaction through reduction in phosphorylated EGFR upon MET knock-down. In LXFA 526 Akt/p70 S6K as well as Erk activation was enhanced after cetuximab treatment despite downregulation of EGFR activity. To elucidate this phenomenon was beyond the scope of this study. Two different hypotheses might explain this. The release of MET from interaction with EGFR, could possibly lead to a more potent downstream signal. Alternately EGFR signalling might confer negative feedback, but not tumour growth in LXFA 526 which is reduced after cetuximab treatment. We propose a model of a signalling network, in which MET is driving tumourigenic growth, but after knock-down or pharmacological inhibition of MET, EGFR comes into play again (Fig. 6C).

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 doi:10.1016/j.ejca.2010.12.019.

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