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Protein biomarkers predictive for response to anti-EGFR treatment in RAS wild-type metastatic colorectal carcinoma

Astrid Lièvre^{1,2,3}, Bérèngère Ouine⁴, Jim Canet⁵, Aurélie Cartier⁴, Yael Amar⁵, Wulfran Cacheux^{6,7}, Odette Mariani⁸, Rosine Guimbaud^{9,10}, Janick Selves^{9,11}, Thierry Lecomte^{12,13}, Serge Guyétant¹⁴, Ivan Bieche⁷, Frédérique Berger^{5,15} and Leanne de Koning^{*,4}

¹Service des maladies de l'appareil digestif, CHU Pontchaillou, 2 rue Henri Le Guilloux, Rennes cedex 09 35033, France; ²Université Rennes 1, Faculté de médecine, 2 Avenue du Prof. Léon Bernard, Rennes 35043, France; ³Inserm ER440-Oncogenesis, Stress and Signaling, Rue Bataille Flandres-Dunkerque, Rennes 35042, France; ⁴Institut Curie, PSL Research University, Department of Translational Research, 26 rue d'Ulm, Paris 75005, France; ⁵Institut Curie, PSL Research University, Unit of Biostatistics, 26 rue d'Ulm, Paris 75005, France; ⁶Institut Curie, Department of Medical Oncology, René Huguenin Hospital, 35 rue Dailly, Saint-Cloud 92210, France; ⁷Institut Curie, Unit of Pharmacogenomics, Department of Genetics, 26 rue d'Ulm, Paris 75005, France; ⁸Institut Curie, PSL Research University, Biological Resource Center, 26 rue d'Ulm, Paris 75005, France; ⁹Centre de Recherche en Cancérologie de Toulouse, Unité Mixte de Recherche, 1037 INSERM—Université Toulouse III, Toulouse 31062, France; ¹⁰Service d'oncologie médicale, Centre Hospitalier Universitaire de Toulouse, Toulouse 31059, France; ¹¹Department of Pathology, Centre Hospitalier Universitaire de Toulouse, Toulouse 31059, France; ¹²Hôpital Trousseau—CHRU de TOURS, Service d'Hépatogastro-Entérologie, Tours 37000, France; ¹³UMR CNRS 7292 (GICC), Université François Rabelais, Tours 37000, France; ¹⁴Hôpital Trousseau—CHRU de TOURS, Service d'Anatomie et Cytologie Pathologiques—Tumorotheque, Tours 37000, France and ¹⁵Institut Curie, PSL Research University, INSERM U900, 26 rue d'Ulm, Paris 75005, France

Background: Metastatic colorectal cancer (mCRC) patients with mutant *KRAS* or *NRAS* are ineligible for anti-epidermal growth factor receptor (anti-EGFR) therapy, as *RAS* mutations activate downstream pathways independently of EGFR and induce primary resistance. However, even among *RAS* wild-type (WT) patients, only a fraction responds to anti-EGFR therapy, suggesting that other mechanisms of resistance exist. We hypothesise that different (epi)genetic alterations can lead to primary anti-EGFR resistance and that the crucial end point is the activation of protein signalling pathways.

Methods: We analysed the expression and activation of proteins involved in cell signalling, using reverse phase protein arrays, on a multicentre French cohort of *RAS* WT mCRC treated with anti-EGFR treatment.

Results: We identify activated EGFR and HER3 as protein biomarkers predictive for better overall survival. Active EGFR signalling and downstream PI3K, but not MAPK, pathway activation are associated with response to anti-EGFR treatment. Left-sided mCRC displays active ErbB2/3 and Wnt pathways and a better response to anti-EGFR therapy compared to right-sided mCRC.

Conclusions: We identify active EGFR and PI3K signalling as a key factor for response to anti-EGFR treatment in mCRC and highlight the importance of developing these biomarkers in clinical practice for the selection of *RAS* WT mCRC patients that would benefit from anti-EGFR treatment.

*Correspondence: Dr L de Koning; E-mail: Leanne.de-koning@curie.fr

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths, with 1.4 million new cases worldwide in 2012. The prognosis of CRC is mainly related to the presence of metastasis: around 25% of patients present with metastasis upon diagnosis and around 50% of patients that are treated for localised CRC will develop metastases during the course of disease. Despite the advances in early diagnosis and treatment achieved in the past 20 years, prognosis of metastatic CRC (mCRC) remains relatively poor, with a 5-year relative survival rate of about 12% (American Cancer Society).

Epidermal growth factor receptor (EGFR or ERBB1) is a cell membrane receptor that belongs to the family of receptor tyrosine kinases (Arteaga and Engelman, 2014). Upon binding of various ligands such as EGF, the receptor is activated and induces the activation of downstream signalling pathways, including PI3K/AKT, MEK/ERK, Jak/Stat and JUNK pathways, which contribute to tumorigenesis. Overexpression or activating mutations of *EGFR* occur in many cancer types, among which CRC. The development of monoclonal antibodies directed against EGFR, such as cetuximab and panitumumab, has significantly improved CRC outcome, both in the context of chemoresistant tumours (Amado *et al*, 2008; Karapetis *et al*, 2008) and as a first-line treatment (Bokemeyer *et al*, 2009; Van Cutsem *et al*, 2009). However, only patients having a tumour without mutations in *KRAS* and *NRAS* can benefit from anti-EGFR therapy (Douillard *et al*, 2013). Indeed, *KRAS* and *NRAS* operate downstream of EGFR in the RAS/MAPK signalling pathway and their mutation activates the pathway independently of *EGFR* status. *KRAS* and *NRAS* mutations are frequent, occurring in around 50% of CRCs, and their sequencing is therefore mandatory before administration of anti-EGFR treatment.

However, even among the patients with wild-type (WT) *KRAS* and *NRAS*, only 20–30% respond to the anti-EGFR treatment monotherapy (Price *et al*, 2016) and 65–70% to anti-EGFR combined with chemotherapy (Heinemann *et al*, 2016), suggesting that other molecular mechanisms of resistance exist. The identification of additional markers of resistance would allow to better select those patients that could benefit from anti-EGFR therapy and avoid inefficient and potentially toxic treatment of the other patients. CRC cell lines, xenografts and, less frequently, patient samples have been used to tackle this question. Multiple studies have shown that activation of the signalling pathways downstream of EGFR, induced by genetic alterations such as *PTEN* deletions, *PIK3CA* mutations or *MET* activation, constitute an important mechanism of primary and acquired resistance towards anti-EGFR (Bardelli and Siena, 2010; Troiani *et al*, 2013; Bajpe *et al*, 2014; Luraghi *et al*, 2014; Song *et al*, 2014; Van Emburgh *et al*, 2014). *HER2* amplification or mutation has also been associated with anti-EGFR resistance in CRC xenografts (Bertotti *et al*, 2011; Yonesaka *et al*, 2011; Bertotti *et al*, 2015). Furthermore, amplifications or mutations of *FGFR1*, *PDGFRA* and *MAP2K1* have been described (Bertotti *et al*, 2015), as well as the deregulation of several microRNAs. Yet, besides *RAS*, no other marker of resistance has been validated so far for clinical practice. In addition, recent data suggest that right-sided mCRC is more resistant to anti-EGFR therapy than left-sided mCRC (Moretto *et al*, 2016; Tejpar *et al*, 2016; Holch *et al*, 2017), but the biology underlying this difference remains elusive.

We hypothesise that many different genetic or epigenetic alterations can be involved in anti-EGFR resistance and that the crucial end point resides in the activation of downstream signalling pathways. The activation of these pathways would thus be a better and more universal predictor of resistance than each genetic alteration separately. However, large-scale protein data on CRC patient samples with clinical follow-up are currently missing. For this reason, we here decided to analyse the expression and the activation of a large panel of proteins involved in cell signalling

pathways, using reverse phase protein arrays (RPPA) on a multicentre French cohort of *RAS* WT mCRCs, both left- and right-sided, treated with anti-EGFR treatment.

MATERIALS AND METHODS

Patient samples. Patients ($n = 53$) with metastatic chemoresistant CRC were treated with anti-EGFR therapy (cetuximab or panitumumab), alone or in association with chemotherapy, at Institut Curie (Paris, France), CHU of Toulouse (France) or CHRU of Tours (France). Patients could be included in the study if tumour response to anti-EGFR could be specifically evaluated, that is, patients treated with a combination of anti-EGFR and chemotherapy who previously progressed on the same chemotherapy component (including those who had progressed on an oxaliplatin-based adjuvant chemotherapy then on a first-line irinotecan-based chemotherapy and those who had progressed on a first-line FOLFIRINOX tritherapy), or patients treated with a monotherapy of anti-EGFR or patients who initially progressed on a first-line combination of anti-EGFR and chemotherapy. According to French regulations, patients were informed of research performed with the biological specimens obtained during their treatment and did not express opposition. This retrospective study was reviewed and approved by the Ethics Committee of the Institut Curie. Time from sample resection to sample freezing was < 30 min in most cases, and always < 60 min. Samples were stored in secured -80°C freezers. For this retrospective study, four 50 μm -thick frozen tissue sections of the primary tumour, obtained before administration of anti-EGFR treatment and containing at least 50% of tumour cells, were sent to the RPPA platform of Curie. Sequencing of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* was performed independently on 3 of the 28 regional molecular genetics platforms constituting the national network of public laboratories dedicated to molecular oncology tests in France that has been certified by the French National Cancer Institute (INCa). According to the INCa quality assurance programme, these platforms have used one of the recommended sequencing techniques with detection sensitivity between 5 and 10% of mutated cells (allelic hybridisation using HRM followed by Sanger sequencing or by pyrosequencing). From the 53 samples, 7 were removed due to low RPPA signals, probably reflecting protein degradation. Twelve more samples had to be excluded from further analysis, because they did not comply with inclusions criteria (6 had a *KRAS* mutation that was not initially determined as these patients were diagnosed before *KRAS* sequencing became a prerequisite for anti-EGFR treatment, for 2 tumours cellularity was too low, 2 patients were responders to a first-line combination of anti-EGFR and chemotherapy so that we could not determine the specific response to the anti-EGFR, and 2 tumours were not CRC). Thus, 34 samples were kept for further analysis.

Reverse phase protein arrays. Samples were disrupted in Laemmli buffer (50 mM Tris (pH = 6.8), 2% SDS, 5% glycerol, 2 mM DTT, 2.5 mM EDTA, 2.5 mM EGTA, $1 \times$ HALT Phosphatase inhibitor (Thermo Scientific, Illkirch, France 78420), Protease inhibitor cocktail complete MINI EDTA-free (Roche, Meylan cedex, France 1836170, 1 tablet per 10 ml), 2 mM Na_3VO_4 and 10 mM NaF), using a Precellys (Bertin, Montigny le Bretonneux, France). Extracts were then boiled for 10 min at 100°C , sonicated to reduce viscosity and centrifuged 10 min at 15 000 r.p.m. The supernatant was collected and stored at -80°C . Protein concentration was determined (BCA reducing agent compatible kit, ref 23252, Pierce, Thermo Scientific). Samples were printed onto nitrocellulose covered slides (Supernova, Grace Biolabs, Bend, OR, USA) using a dedicated arrayer (2470 arrayer, Aushon Biosystems, Billerica, MA, USA). Five serial dilutions, ranging from 1500 to $94 \mu\text{g ml}^{-1}$, and three

technical replicates per dilution were printed for each sample. Arrays were labelled with 86 specific antibodies (see Supplementary Table 1 for a complete list of antibody references) or without primary antibody (negative control), using an Autostainer Plus (Dako, Les Ulis, France). Briefly, slides were incubated with avidin, biotin and peroxidase-blocking reagents (Dako) before saturation with TBS containing 0.1% Tween-20 and 5% BSA (TBST-BSA). Slides were then probed overnight at 4 °C with primary antibodies diluted in TBST-BSA. After washes with TBST, arrays were probed with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in TBST-BSA for 1 h at RT. To amplify the signal, slides were incubated with Bio-Rad Amplification Reagent (Bio-Rad, Marnes-la-Coquette, France) for 15 min at RT. The arrays were washed with TBST, probed with Alexa647-Streptavidin (Molecular Probes, Thermo Scientific) diluted in TBST-BSA for 1 h at RT and washed again in TBST. For staining of total protein, arrays were incubated 15 min in 7% acetic acid and 10% methanol, rinsed twice in water, incubated 10 min in Sypro Ruby (Invitrogen, Thermo Scientific) and rinsed again. The processed slides were dried by centrifugation and scanned using a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA). Spot intensity was determined with MicroVigene software (VigeneTech Inc., Carlisle, MA, USA). All primary antibodies used in RPPA have been previously tested by western blotting to assess their specificity for the protein of interest.

Samples with low overall signal or with aberrant dilution curves, which are often indicative of protein degradation, were discarded during quality control. For each antibody, the median signal intensity was at least three times higher than the background array without primary antibody. Raw data were normalised using Normacurve (Troncale *et al*, 2012), which normalises spot-wise for a negative control slide (labelled without any primary antibody, provides the fluorescent background) and a slide labelled with a total protein stain (serves as a loading control). Next, Normacurve draws the antibody response curve for each array. Each sample, including the five serial dilutions and the replicates, is aligned onto this curve to calculate one normalised value per sample. These normalised values are then used for statistical analysis.

Statistical analysis. Response to anti-EGFR treatment was determined using RECIST criteria (Therasse *et al*, 2000). Continuous variables are described as mean and s.d.'s and qualitative data are presented as a number and percentage of sample size. The association between clinical variables and proteins was determined by Wilcoxon or Kruskal–Wallis tests. Association between discrete variables was tested using χ^2 - or Fisher's exact test. Hierarchical clustering was performed using Ward metrics and Pearson correlation and represented as a heatmap.

Protein expression was divided in high expression and low expression with the cutoff at the median expression level.

The RECIST criteria were restricted to a dichotomous output, where complete or partial response were considered as a response and stabilisation or progression as a non-response. Univariate logistic regression analysis was performed and odds ratios (ORs) were calculated to measure the association between the expression level of a protein and the chance to respond to treatment. A multivariate logistic regression analysis was performed including significant proteins ($P < 0.05$) with a stepwise procedure.

Overall survival is defined as the time between diagnosis of the metastasis and the date of death. Patients still alive at the moment of analysis were censored at the date of last follow-up. Survival curves were estimated using Kaplan–Meier and compared with log-rank tests.

Univariate and multivariate Cox proportional hazard models were performed to determine the variables that impacts OS. Only

variables with a significant P -value ($P < 0.05$) were included in a multivariate stepwise procedure using the Cox model.

P -values below 0.05 were considered significant. All the analyses were performed using R software version 3.3.0 (R Core Team, 2015).

RESULTS

Patient characteristics and response to anti-EGFR therapy according to primary tumour location. Final RPPA data were obtained for 34 CRCs and 86 antibodies. The antibodies were selected according to signalling pathways that have been put forward as being involved in EGFR signalling and anti-EGFR resistance (various RTKs, PI3K/Akt pathway and MAPK pathways) or more generally in CRC carcinogenesis (Wnt/Notch) and chemotherapy response (apoptosis, cell proliferation, DNA repair and angiogenesis). Clinical characteristics are summarised in Table 1 and antibodies are listed in Supplementary Table 1. Median follow-up of these 34 patients was 15.5 months from the beginning of anti-EGFR treatment (range: 3–45.6 months). All patients received anti-EGFR as second- or third-line treatment, except for 4 patients who received anti-EGFR in combination with

Table 1. Description of clinical variables of mCRC samples.

	N	%
Sex		
Male	20	58.8
Female	14	41.4
Age at diagnosis		
<50	11	32.4
≥50	23	67.6
Localisation		
Colon right-sided	10	29.4
Colon left-sided	15	44.1
Rectum	9	26.5
Treatment		
Cetuximab	20	58.8
Panitumumab	14	41.2
Associated chemotherapy		
Monotherapy	4	11.8
Irinotecan	5	14.8
FOLFIRI	21	61.8
FOLFOX	4	11.8
RECIST score		
Complete response	0	0.00
Partial response	11	32.4
Stabilisation	11	32.4
Progression	12	35.3
Line of treatment		
First line	4	11.8
Second line	13	38.2
Third line	17	50.0
Number of metastatic sites		
1	12	35.3
2	13	38.2
3	9	26.5
Mutations in PIK3CA		
WT	29	85.3
Mutant	5	14.7
Mutations in BRAF		
WT	33	97.1
Mutated	1	2.9

Abbreviations: mCRC = metastatic colorectal cancer; WT = wild type.

chemotherapy as a first-line treatment but did not respond. All patients of this cohort were thus shown to be chemoresistant, and any observed response to therapy could be attributed solely to the addition of anti-EGFR treatment. In all, 11 patients showed a partial response, 11 patients showed stabilisation and 12 patients showed disease progression. No complete response was observed. Among the 11 patients with stabilisation, the median time to progression was 8 months (range: 3–13 months) vs 16 months (range: 5–46 months) in patients with partial response and 2 months (range: 1–3 months) in patients with progressive disease. Given the small sample size of patients with stabilisation, we did not perform a further categorisation of stabilised patients according to duration of stabilisation, which would limit the significance of the results. In all, 5 patients showed a *PIK3CA* hot spot activating mutation (3 patients have p.E545K, 1 patient p.E542K and 1 patient p.N1044K) and 1 other patient a *BRAF* V600E mutation. Biopsies were obtained before initiation of the anti-EGFR therapy.

We analysed whether the response to treatment, measured according to RECIST criteria, was associated with any of the clinical parameters. The RECIST score was not significantly different according to sex (men vs female), age (<50 vs ≥50) or the molecule of anti-EGFR treatment (cetuximab vs panitumumab)

that was administered. As expected, the absence of objective response was associated to worse overall survival (data not shown). Interestingly, and in accordance with recent findings (Moretto *et al*, 2016; Tejpar *et al*, 2016; Holch *et al*, 2017) the response to anti-EGFR treatment was better in left-sided CRC (descending colon and rectum), where 46% of patients show partial response, than in right-sided CRC (ascending colon), where none of the patients shows partial response ($P = 0.03$).

Protein expression according to patient and tumour characteristics. We studied whether certain proteins, measured by RPPA, were associated with clinical parameters. None of the measured proteins showed a significant association with sex, age or the number of metastatic sites (1 vs ≥2). Next, we compared the protein profiles of left-sided vs right-sided CRC. Out of 86 protein analysed, 76 proteins are differentially expressed or activated between the two locations ($P < 0.05$), demonstrating an important difference in terms of signalling pathway activation. The phosphorylated proteins that are differentially expressed between left- and right-sided CRC are enriched in HER2/HER3 signalling ($P = 0.014$) and in the Wnt pathway ($P = 0.018$; Figure 1A), which both seem to be more activated in left-sided CRC as previously suggested (Kim *et al*, 2015; Figure 1B and C).

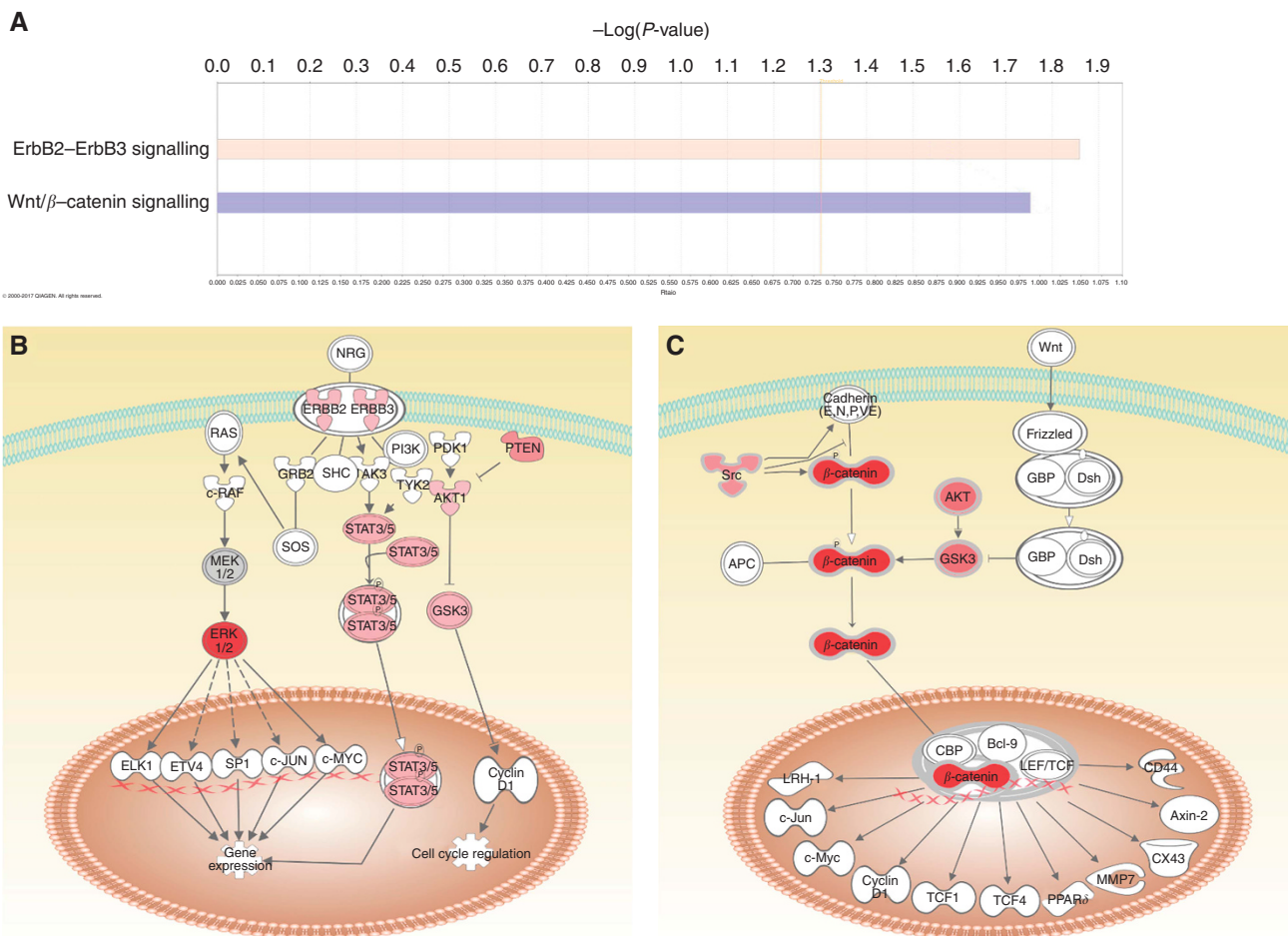


Figure 1. Differences in pathway activation between left-sided vs right-sided CRC. **(A)** Phosphorylated proteins that are differentially expressed ($P < 0.05$) between left-sided and right-sided CRC were analysed using Ingenuity Pathway Analysis and found to be enriched in ErbB and Wnt signalling. Enrichment is calculated against the list of analysed proteins. The indicated threshold of $-\log(P\text{-value}) = 1.3$ corresponds to $P = 0.05$. Expression data were overlaid on a schematic representation of the ErbB **(B)** and Wnt pathways **(C)** showing a higher activation of all measured proteins in left-sided colon. The red colour gradient of the proteins represents the fold change between left- and right-sided CRC, with a darker colour indicating a greater fold-change. White proteins are part of the pathway but have not been analysed in this project.

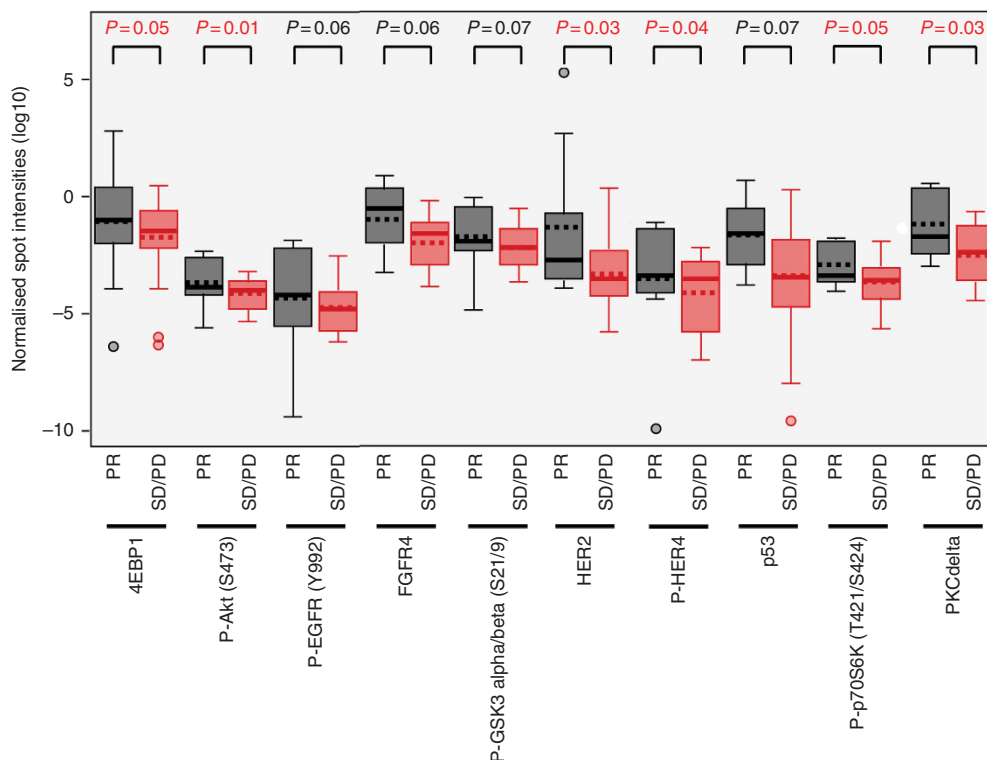


Figure 2. Distribution of (phospho-)proteins that are differentially expressed according to the response to anti-EGFR treatment as measured with the RECIST criteria: comparison of stable disease + progressive disease vs partial response (PR). No complete response was observed in our study. *P*-values are indicated above each comparison. Only protein biomarkers with a *P*-value of ≤ 0.07 are shown. Boxes contain 50% of samples, horizontal line represents the median and isolated dots represent outliers.

Protein biomarkers predictive for response to anti-EGFR therapy. Using the RECIST criteria, we searched for (phospho-)proteins associated with response to anti-EGFR antibodies that could thus constitute potential predictive biomarkers. Because of small group sizes and to better identify biomarkers the most predictive of response to anti-EGFR, we compared partial response ($n = 11$) vs stabilisation + progression ($n = 23$). Higher levels of Phospho-Akt (Ser473) ($P = 0.01$), HER2 ($P = 0.03$), PKC delta ($P = 0.03$), phospho-HER4 ($P = 0.04$), phospho-p70S6kinase ($P = 0.05$) and 4EBP1 ($P = 0.05$) are associated with response to treatment in univariate analyses (Figure 2). In addition, several proteins show a trend towards significance: higher levels of phospho-EGFR ($P = 0.06$), FGFR4 ($P = 0.06$), phospho-GSK3 ($P = 0.07$) and p53 ($P = 0.07$) are associated with a better response to treatment (Figure 2). Multivariate analyses allow evidencing phospho-Akt (Ser473) as the dominating biomarker for response (OR = 5.5, CI 95% (1.6; 34.2)).

Prognostic factors associated with survival. Next, we studied which characteristics were associated with overall survival, defined as the time between the diagnosis of the metastasis and death. Using unsupervised hierarchical clustering, the 86 analysed proteins allow a separation of the 34 CRCs into two clearly distinguishable groups (Supplementary Figure 1). The two clusters do not separate the patients according to response, location of the primary tumour (left- or right-sided, or rectum vs colon) or the centre of origin of the samples. In addition, the two clusters do not display a significant difference in survival (data not shown). Left-sided CRC seem to have a better overall survival compared to right-sided CRC (survival rate at 24 months of 78% (CI 95% (63%; 98%)) and 36% (CI 95% (13%; 99%)) respectively), although the log-rank test is not significant (Supplementary Figure 2).

Next, we studied which individual (phospho-)proteins are associated with overall survival. We found that a high expression

of EGFR ($P = 0.01$), phospho-EGFR (Tyr1173) ($P = 0.03$) and HER3 ($P = 0.03$) is associated with a better survival (Figure 3). The expression of phospho-EGFR and HER3 proteins is highly correlated (correlation coefficient $R = +0.82$, $P < 0.001$) and they show identical survival curves (Figure 3B and C). Both proteins also correlate with total EGFR ($R = 0.62$ and 0.61 respectively; $P < 0.001$). Higher expression of FGFR3 ($P = 0.05$), phospho-4EBP1 ($P = 0.05$), p53 ($P = 0.06$) and phospho-HER3 ($P = 0.06$) shows a trend towards an association with better survival, without reaching significance.

Correlation between mutational data, response to treatment and protein expression. In our cohort, a single patient had a *BRAF* V600E mutation and had progressive disease. Five patients had *PIK3CA* mutations. From these five patients, three had a partial response; one had a stable disease and one a progressive disease. Although the numbers are too small for statistical analysis, there is thus no indication in our cohort that *PIK3CA* mutation associates with anti-EGFR resistance. Despite the lack of statistical power, we addressed whether the five *PIK3CA*-mutated tumours indeed show a higher activation of the Akt pathway than *PIK3CA* WT tumours. The ratios of phospho-Akt (Thr308)/Akt, of phospho-p70S6K/p70S6K and of P-PKC α /PKC α (a downstream target of PDK1 and mTOR) were slightly increased when *PIK3CA* is mutated ($P = 0.11$, 0.14 and 0.10, respectively), without reaching significance though (Supplementary Figure 3).

DISCUSSION

Only a fraction of mCRC patients having WT *KRAS* and *NRAS* benefit from anti-EGFR treatment, suggesting the presence of additional molecular characteristics leading to primary resistance. Many genetic alterations have been shown to induce resistance in

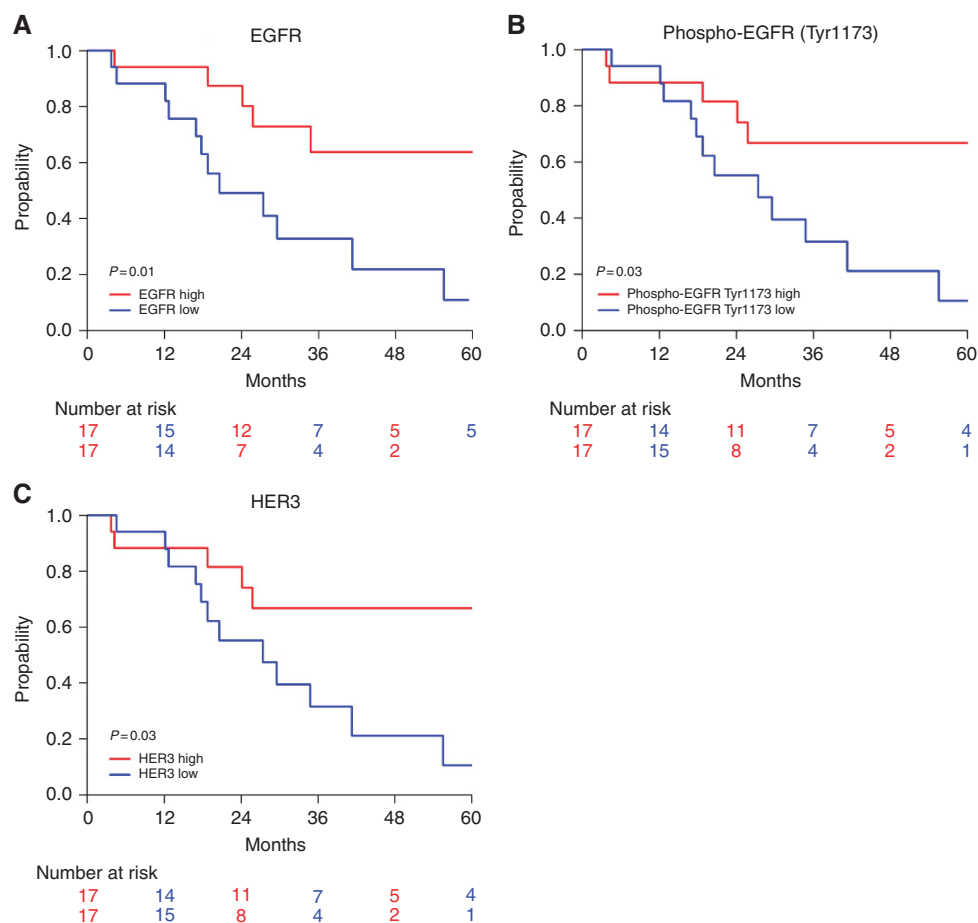


Figure 3. (Phospho-)proteins associated with overall survival. Kaplan–Meier curves of overall survival according to expression levels of EGFR (A), phospho-EGFR (Tyr1173) (B) and HER3 (C). Red line: expression higher than the median expression level; blue line: expression lower than the median. The result of the log-rank test is indicated in each graph and the patients at risk over time are indicated below each graph.

cell lines or xenograft models. Here we hypothesised that all these alterations will ultimately lead to the activation of cell signalling pathways that can be measured at the protein level.

We performed the largest targeted proteomics study published so far in terms of analysed proteins on a small but well annotated cohort of 34 *KRAS* and *NRAS* WT mCRC samples from patients that received anti-EGFR therapy, with the aim to identify predictive markers of sensitivity or resistance.

We identified several (phospho-)proteins that are predictive for response to treatment or for overall survival in mCRC patients. Although the observed differences per protein are small and not highly significant due to the small study size, the identified proteins reveal interesting patterns.

Indeed, we show that independently of the line of treatment, patients with higher expression and activation of EGFR and HER3 membrane receptors have a better overall survival. HER3, which lacks a functional kinase domain, heterodimerises with EGFR or with HER2 to produce a potent signalling complex (Jura *et al*, 2009). Targeting EGFR and HER3 concomitantly is a current lead in CRC (Juric *et al*, 2015; Temraz *et al*, 2016). Thus, actively signalling EGFR is associated with a better overall survival in these patients receiving anti-EGFR treatment, probably because these tumours are more dependent on EGFR signalling and thus more sensitive to its inhibition.

If we look more specifically at the response to treatment, as defined by the RECIST criteria, a broader picture appears. The proteins that associate with a better response to therapy are mostly associated with an activation of tyrosine kinases (EGFR, HER2,

HER4 and FGFR4) and the downstream signalling through the PI3K/Akt/mTOR pathway (GSK3 and 4EBP1, which are downstream of Akt; p70S6K, which is downstream of mTOR; and PKC Ω , which can be activated by EGFR), thus revealing a complex regulatory network (Figure 4). Thus, patients with activated cell surface receptors (notably EGFR) and PI3K pathway are more likely to respond to anti-EGFR therapy. Our data confirm and extend previously reported observations on the predictive value of phospho-EGFR and phospho-Akt (Van Schaeuybroeck *et al*, 2005; Harle *et al*, 2015).

In CRC carrying a mutation in *KRAS* or *NRAS*, this mutation confers resistance to anti-EGFR treatment by activating MAPK and PI3K pathways. In our patient cohort of *RAS* WT CRCs, we observe active EGFR signalling and downstream PI3K pathway activation. Interestingly, we do not identify components of the MAPK pathway as being predictive for response to treatment, suggesting that in *RAS* WT patients the PI3K pathway is the predominant pathway that explains variability in response to anti-EGFR therapies. Several potential mechanisms could be at the origin of the EGFR activation in these *RAS* WT tumours. First, the overexpression of EGFR ligands and notably epiregulin and amphiregulin, which activate EGFR, has been associated with a better response to anti-EGFR therapy in *RAS* WT CRC (Khambata-Ford *et al*, 2007; Baker *et al*, 2011; Jonker *et al*, 2014; Seligmann *et al*, 2016). Second, mutations in genes such as *PIK3CA*, *PTEN*, *EGFR* and *ERBB2*, were recently found predictive for anti-EGFR therapy in 31% of *RAS* WT tumours (Rankin *et al*, 2016). In our cohort, mutation status was determined for *BRAF*

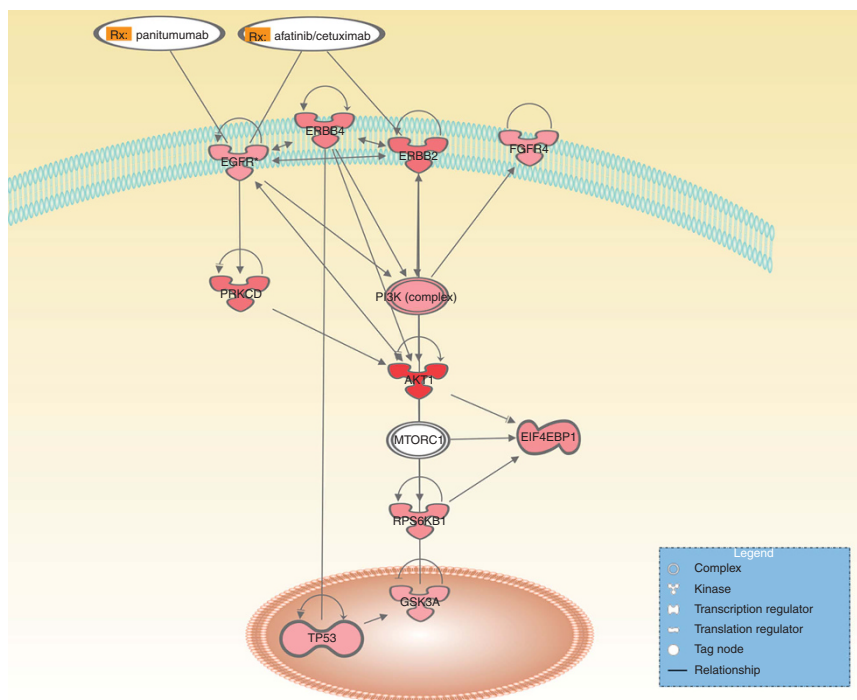


Figure 4. Simplified scheme of signalling interaction network between the proteins that are associated with response to anti-EGFR therapy. The red colour gradient reflects the *P*-value with a darker colour indicating a lower *P*-value. Only direct and experimentally proven interactions between proteins, described in literature, are shown.

and *PIK3CA*. A single patient had a *BRAF*-mutated tumour and had progressive disease as expected. Indeed, the V600E mutation in the gene that encodes *BRAF*, which acts downstream of *RAS*, is known to confer resistance to anti-EGFR therapy and a very poor prognosis of CRC patients (Pietrantonio *et al*, 2015; Rowland *et al*, 2015). *PIK3CA* mutations were previously also suggested to lead to resistance to anti-EGFR therapy (Sartore-Bianchi *et al*, 2009), although large-scale meta-analyses suggest that this is true only for mutations in exon 20 and not for mutations in exon 9 (De Roock *et al*, 2010). We detected activating *PIK3CA* hot spot mutations in five patients: four mutations in exon 9 (three p.E545K and one p.E542K mutation) and one in exon 20 (p.N1044K). From these five patients, three had a partial response (including the patient with the exon 20 mutated tumour), one had a stable disease and one a progressive disease. Although the numbers are too small for statistical analysis, there is thus no indication in our cohort that *PIK3CA* mutation associates with anti-EGFR resistance. On the contrary, *PIK3CA* mutations could be one method to activate the PI3K pathway, which we find associated with a better response to therapy.

During the course of our study, large-scale meta-analyses revealed that left-sided and right-sided CRC do not respond similarly to anti-EGFR therapy (Moretto *et al*, 2016; Tejpar *et al*, 2016; Holch *et al*, 2017). We here confirm that left-sided CRC has a better response to anti-EGFR therapy, a tendency towards better survival and a very different profile of protein expression with notably more Wnt and ErbB signalling activation.

In conclusion, we identified activated EGFR and HER3 as biomarkers predictive for a better overall survival in patients treated by anti-EGFR therapy. Response to treatment, on the other hand, was associated with several markers that converge to active EGFR signalling and in particular the PI3K pathway. Validation of these markers by immunohistochemistry on a large panel of samples would therefore be a crucial step forwards to improved patient stratification and personalised medicine in *RAS* WT CRC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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