

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

Astrid Lièvre, Bérèngere Ouine, Jim Canet, Aurélie Cartier, Yael Amar, Wulfran Cacheux, Odette Mariani, Rosine Guimbaud, Janick Selves, Thierry Lecomte, et al.

▶ **To cite this version:**

Astrid Lièvre, Bérèngere Ouine, Jim Canet, Aurélie Cartier, Yael Amar, et al.. Protein biomarkers predictive for response to anti-EGFR treatment in RAS wild-type metastatic colorectal carcinoma. British Journal of Cancer, 2017, 117 (12), pp.1819-1827. 10.1038/bjc.2017.353 . hal-01682154

**HAL Id: hal-01682154**

**https://univ-rennes.hal.science/hal-01682154**

Submitted on 17 Jan 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Title:

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

Running Title: Proteins predicting anti-EGFR response in mCRC

**Authors:**

Astrid Lièvre [1, 2]

Berengere Ouine [3]

Jim Canet [4]

Aurélie Cartier [3]

Yael Amar [4]

Wulfran Cacheux [5, 12]

Odette Mariani [6]

Rosine Guimbaud [7]

Janick Selves [8]

Thierry Lecomte [9, 10]

Serge Guyetant [11]

Ivan Bieche [12]

Frédérique Berger [4, 13]

Leanne de Koning [3]

**Affiliations :**

1 Service des maladies de l'appareil digestif, CHU Pontchaillou, 2 rue Henri Le Guilloux, 35033 Rennes cedex 09, France; Université Rennes 1, Faculté de médecine, 2 Avenue du Prof. Léon Bernard, 35043 Rennes, France

2 Inserm ER440-Oncogenesis, Stress and Signaling, Rue Bataille Flandres-Dunkerque, 35042 Rennes, France.

3 Institut Curie, PSL Research University, Department of Translational Research, 26 rue d'Ulm, Paris, France

4 Institut Curie, PSL Research University, Unit of biostatistics, 26 rue d'Ulm, Paris, France

5 Institut Curie, Department of medical oncology, René Huguenin Hospital, 35 rue Dailly, 92210 Saint-Cloud, France.

6 Institut Curie, PSL Research University, Biological Resource Center, 26 rue d'Ulm, Paris, France

7 Centre de Recherche en Cancérologie de Toulouse, Unité Mixte de Recherche, 1037 INSERM - Université Toulouse III, Toulouse, France; Service d'oncologie médicale, Centre Hospitalier Universitaire de Toulouse, Toulouse, France

8 Centre de Recherche en Cancérologie de Toulouse, Unité Mixte de Recherche, 1037 INSERM - Université Toulouse III, Toulouse, France; Department of Pathology, Centre Hospitalier Universitaire de Toulouse, Toulouse, France.

9 Hôpital Trousseau - CHRU de TOURS, Service d'Hépato-Gastro-Entérologie, TOURS, France

10 UMR CNRS 7292 (GICC), Université François Rabelais, Tours

11 Hôpital Trousseau - CHRU de TOURS, Service d'Anatomie et Cytologie Pathologiques - Tumorothèque, TOURS, France

12 Institut Curie, Unit of pharmacogenomics, Department of Genetics, 26 rue d'Ulm, Paris, France

13 Institut Curie, PSL Research University , INSERM U900, 26 rue d'Ulm, Paris, France

Corresponding author: Leanne de Koning, Institut Curie, Department of Translational Research, 75005 Paris, France. E-mail: Leanne.de-koning@curie.fr

## Abstract

**Background**: Metastatic colorectal cancer (mCRC) patients with mutant *KRAS* or *NRAS* are ineligible for anti-EGFR therapy, since *RAS* mutations activate downstream pathways independently of EGFR and induce primary resistance. However, even among *RAS* wildtype patients, only a fraction responds to anti-EGFR therapy, suggesting that other mechanisms of resistance exist. We hypothesize that different (epi)genetic alterations can lead to primary anti-EGFR resistance and that the crucial endpoint is the activation of protein signaling pathways.

**Methods**: We analyzed the expression and activation of proteins involved in cell signaling, using Reverse Phase Protein Arrays, on a multi-center French cohort of *RAS* wildtype mCRC treated with anti-EGFR treatment.

**Results**: We identify activated EGFR and HER3 as protein biomarkers predictive for better overall survival. Active EGFR signaling 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.

**Conclusion**: We identify active EGFR and PI3K signaling 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* wildtype mCRC patients that would benefit from anti-EGFR treatment.

**Keywords:** colorectal cancer, resistance, EGFR, biomarkers, cell signaling, RPPA, PI3K

## Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths, with 1.4 million new cases world-wide 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 localized 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 signaling 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 tumors (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 tumor 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 signaling 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 wildtype *KRAS* and *NRAS*, only 20 to 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 signaling 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*, *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 hypothesize 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 signaling 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 is currently missing. For this reason, we here decided to analyze the expression and the activation of a large panel of proteins involved in cell signaling pathways, using Reverse Phase Protein Arrays (RPPA) on a multi-center French cohort of *RAS* wild-type mCRCs, both left- and right-sided, treated with anti-EGFR treatment.

## Methods and materials

*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 tumor response to anti-EGFR could be specifically evaluated i.e. 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 less than 30 minutes in most cases, and always less than 60 minutes. Samples were stored in secured -80°C freezers. For this retrospective study, four 50µm thick frozen tissue sections of the primary tumor, obtained before administration of anti-EGFR treatment and containing at least 50% of tumor 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 program, these platforms have used one of the recommended sequencing techniques with detection sensitivity between 5% and 10% of mutated cells (allelic

hybridization 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. 12 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 since these patients were diagnosed before KRAS sequencing became a prerequisite for anti-EGFR treatment, for 2 tumors 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 tumors 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, 1x HALT Phosphatase inhibitor (Perbio 78420), Protease inhibitor cocktail complete MINI EDTA-free (Roche 1836170, 1 tablet/10 mL), 2 mM Na3VO4 and 10 mM NaF), using a Precellys (Bertin). Extracts were then boiled for 10 min at 100°C, sonicated to reduce viscosity and centrifuged 10 min at 15000 rpm. The supernatant was harvested and stored at -80°C. Protein concentration was determined (Pierce BCA reducing agent compatible kit, ref 23252). Samples were printed onto nitrocellulose covered slides (Supernova, Grace Biolabs) using a dedicated arrayer (2470 arrayer, Aushon Biosystems). Five serial dilutions, ranging from 1500 to 94 µg/ml, and three technical replicates per dilution were printed for each sample. Arrays were labeled 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). 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) diluted in TBST-BSA for 1h at RT. To amplify the signal, slides were incubated with Bio-Rad Amplification Reagent for 15 min at RT. The arrays were washed with TBST, probed with Alexa647-Streptavidin (Molecular Probes) diluted in TBST-BSA for 1h 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) and rinsed again. The processed slides were dried by centrifugation and scanned using a GenePix 4000B microarray scanner (Molecular Devices). Spot intensity was determined with MicroVigene software (VigeneTech Inc). 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 normalized using Normacurve (Troncale *et al.* 2012), which normalizes spot-wise for a negative control slide (labeled without any primary antibody, provides the fluorescent background) and a slide labeled 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, are aligned onto this curve to calculate one normalized value per sample. These normalized 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 standard deviations 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 Chi-square 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 cut-off 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 stabilization or progression as a non-response. Univariate logistic regression analysis was performed and odds ratios 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 censured 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 tumor location*

Final RPPA data was obtained for 34 CRCs and 86 antibodies. The antibodies were selected according to signaling pathways that have been put forward as being involved in EGFR signaling and anti-EGFR resistance (various RTKs, PI3K/Akt pathway, MAPK pathways) or more generally in CRC carcinogenesis (Wnt/Notch) and chemotherapy response (apoptosis, cell proliferation, DNA repair, angiogenesis). Clinical characteristics are summarized 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 four patients who received anti-EGFR in combination with 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. 11 patients showed a partial response, 11 patients showed stabilization and 12 patients showed disease progression. No complete response was observed. Among the 11 patients with stabilization, the median time to progression was 8 months (range: 3-13 month) versus 16 months (range: 5-46) in patients with partial response and 2 months (range:1-3 months) in patients with progressive disease respectively. Given the small sample size of patients with stabilization, we did not perform a further categorization of stabilized patients according to duration of stabilization, which would limit the significance of the results. Five patients showed a *PIK3CA* hot spot activating mutation (3 patients have p.E545K, one patient p.E542K and one patient p.N1044K) and one other patient a *BRAF* V600E mutation. Biopsies were obtained before initiation of the anti-EGFR therapy.

We analyzed if 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

versus female), age (<50 versus ≥50) or the molecule of anti-EGFR treatment (cetuximab versus 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 tumor characteristics*

We studied if 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 versus right-sided CRC. Out of 86 protein analyzed, 76 proteins are differentially expressed or activated between the two locations (p<0.05), demonstrating an important difference in terms of signaling pathway activation. The phosphorylated proteins that are differentially expressed between left and right sided CRC are enriched in HER2/HER3 signaling (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 1C).

*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) versus stabilization + 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, CI95% [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 analyzed 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 tumor (left- or right-sided, or rectum versus colon) or the center 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% (CI95% [63% ; 98%]) and 36% (CI95%[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) are 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 3C). Both proteins also correlate with total EGFR (R= 0.62 and R= 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) show 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 5 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 if the 5 *PIK3CA* mutated tumors indeed show a higher activation of the Akt pathway than *PIK3CA* WT tumors. The ratios of Phospho-Akt (Thr308)/Akt, of phospho-p70S6K/p70S6K and of P-PKCα/PKCα (a downstream target of PDK1 and mTOR) were slightly increased when *PIK3CA* is mutated (p=0.11; p=0.14 and p=0.10, respectively), without reaching significance though (Supplementary Figure 3).

## Discussion

Only a fraction of mCRC patients having wild-type *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 cell lines or xenograft models. Here, we hypothesized that all these alterations will ultimately lead to the activation of cell signaling pathways that can be measured at the protein level.

We performed the largest targeted proteomics study published so far in terms of analyzed 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, heterodimerizes with EGFR or with HER2 to produce a potent signaling 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 signaling EGFR is associated with a better overall survival in these patients receiving anti-EGFR treatment, probably because these tumors are more dependent on EGFR signaling 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 signaling through the PI3K/Akt/mTOR pathway (GSK3 and 4EBP1 which are downstream of Akt, p70S6K which is

downstream of mTOR, PKCɗ 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 extent previously reported observations on the predictive value of Phospho-EGFR and Phospho-Akt (Van Schaeybroeck *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 signaling 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 tumors. 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 tumors (Rankin *et al.* 2016). In our cohort, mutation status was determined for *BRAF* and *PIK3CA*. A single patient had a *BRAF*-mutated tumor 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 5 patients: 4 mutations in exon 9 (three p.E545K and one p.E542K mutation) and one in exon 20 (p.N1044K). From these 5 patients, three had a partial response (including

the patient with the exon 20 mutated tumor), 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 signaling 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 signaling 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 personalized medicine in *RAS* wildtype CRC.

## Acknowledgements

We thank Stéphane Liva and Patrick Poullet for bioinformatics support. This work was financed by the Institut Curie Evaluation Committee of Translational Research (CEST) and Institut Carnot Curie-Cancer 2013.

## Conflict of Interest statement

The authors have no conflicts of interest to declare.

## References


Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD and Chang DD (2008). Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol. 26(10): 1626-1634. doi: 1610.1200/JCO.2007.1614.7116. Epub 2008 Mar 1623.

American Cancer Society. "https://www.cancer.org."

Arteaga CL and Engelman JA (2014). ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. Cancer Cell. 25(3): 282-303. doi: 210.1016/j.ccr.2014.1002.1025.

Bajpe PK, Prahallad A, Horlings H, Nagtegaal I, Beijersbergen R and Bernards R (2014). A chromatin modifier genetic screen identifies SIRT2 as a modulator of response to targeted therapies through the regulation of MEK kinase activity. Oncogene 27(10): 588.

Baker JB, Dutta D, Watson D, Maddala T, Munneke BM, Shak S, Rowinsky EK, Xu LA, Harbison CT, Clark EA, Mauro DJ and Khambata-Ford S (2011). Tumour gene expression predicts response to cetuximab in patients with KRAS wild-type metastatic colorectal cancer. Br J Cancer 104(3): 488-495.

Bardelli A and Siena S (2010). Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol. 28(7): 1254-1261. doi: 1210.1200/JCO.2009.1224.6116. Epub 2010 Jan 1225.

Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, Cora D, Di Nicolantonio F, Buscarino M, Petti C, Ribero D, Russolillo N, Muratore A, Massucco P, Pisacane A, Molinaro L, Valtorta E, Sartore-Bianchi A, Risio M, Capussotti L, Gambacorta M, Siena S, Medico E, Sapino A, Marsoni S, Comoglio PM, Bardelli A and Trusolino L (2011). A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. Cancer Discov 1(6): 508-523.

Bertotti A, Papp E, Jones S, Adleff V, Anagnostou V, Lupo B, Sausen M, Phallen J, Hruban CA, Tokheim C, Niknafs N, Nesselbush M, Lytle K, Sassi F, Cottino F, Migliardi G, Zanella ER, Ribero D, Russolillo N, Mellano A, Muratore A, Paraluppi G, Salizzoni M, Marsoni S, Kragh M, Lantto J, Cassingena A, Li QK, Karchin R, Scharpf R, Sartore-Bianchi A, Siena S, Diaz LA, Jr., Trusolino L and Velculescu VE (2015). The genomic landscape of response to EGFR blockade in colorectal cancer. Nature 526(7572): 263-267.

Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh C, Loos AH, Zubel A and Koralewski P (2009). Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol. 27(5): 663-671. doi: 610.1200/JCO.2008.1220.8397. Epub 2008 Dec 1229.

De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M and Tejpar S (2010). Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11(8): 753-762.

Douillard JY, Rong A and Sidhu R (2013). RAS mutations in colorectal cancer. N Engl J Med 369(22): 2159-2160.

Harle A, Salleron J, Perkins G, Pilati C, Blons H, Laurent-Puig P and Merlin JL (2015). Expression of pEGFR and pAKT as response-predictive biomarkers for RAS wild-type patients to anti-EGFR monoclonal antibodies in metastatic colorectal cancers. Br J Cancer 113(4): 680-685.

Heinemann V, Rivera F, O'Neil BH, Stintzing S, Koukakis R, Terwey JH and Douillard JY (2016). A study-level meta-analysis of efficacy data from head-to-head first-line trials of epidermal growth factor

receptor inhibitors versus bevacizumab in patients with RAS wild-type metastatic colorectal cancer. Eur J Cancer 67: 11-20.
Holch JW, Ricard I, Stintzing S, Modest DP and Heinemann V (2017). The relevance of primary tumour location in patients with metastatic colorectal cancer: A meta-analysis of first-line clinical trials. Eur J Cancer. 70:87-98.(doi): 10.1016/j.ejca.2016.1010.1007. Epub 2016 Nov 1029.
Jonker DJ, Karapetis CS, Harbison C, O'Callaghan CJ, Tu D, Simes RJ, Malone DP, Langer C, Tebbutt N, Price TJ, Shapiro J, Siu LL, Wong RP, Bjarnason G, Moore MJ, Zalcberg JR and Khambata-Ford S (2014). Epiregulin gene expression as a biomarker of benefit from cetuximab in the treatment of advanced colorectal cancer. Br J Cancer 110(3): 648-655.
Jura N, Shan Y, Cao X, Shaw DE and Kuriyan J (2009). Structural analysis of the catalytically inactive kinase domain of the human EGF receptor 3. Proc Natl Acad Sci U S A 106(51): 21608-21613.
Juric D, Dienstmann R, Cervantes A, Hidalgo M, Messersmith W, Blumenschein GR, Jr., Tabernero J, Roda D, Calles A, Jimeno A, Wang X, Bohorquez SS, Leddy C, Littman C, Kapp AV, Shames DS, Penuel E, Amler LC, Pirzkall A and Baselga J (2015). Safety and Pharmacokinetics/Pharmacodynamics of the First-in-Class Dual Action HER3/EGFR Antibody MEHD7945A in Locally Advanced or Metastatic Epithelial Tumors. Clin Cancer Res. 21(11): 2462-2470. doi: 2410.1158/1078-0432.CCR-2414-2412.
Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ and Zalcberg JR (2008). K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med. 359(17): 1757-1765. doi: 1710.1056/NEJMoa0804385.
Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, Wong TW, Huang X, Takimoto CH, Godwin AK, Tan BR, Krishnamurthi SS, Burris HA, 3rd, Poplin EA, Hidalgo M, Baselga J, Clark EA and Mauro DJ (2007). Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 25(22): 3230-3237.
Kim SE, Paik HY, Yoon H, Lee JE, Kim N and Sung MK (2015). Sex- and gender-specific disparities in colorectal cancer risk. World J Gastroenterol 21(17): 5167-5175.
Luraghi P, Reato G, Cipriano E, Sassi F, Orzan F, Bigatto V, De Bacco F, Menietti E, Han M, Rideout WM, 3rd, Perera T, Bertotti A, Trusolino L, Comoglio PM and Boccaccio C (2014). MET signaling in colon cancer stem-like cells blunts the therapeutic response to EGFR inhibitors. Cancer Res. 74(6): 1857-1869. doi: 1810.1158/0008-5472.CAN-1813-2340-T. Epub 2014 Jan 1821.
Moretto R, Cremolini C, Rossini D, Pietrantonio F, Battaglin F, Mennitto A, Bergamo F, Loupakis F, Marmorino F, Berenato R, Marsico VA, Caporale M, Antoniotti C, Masi G, Salvatore L, Borelli B, Fontanini G, Lonardi S, De Braud F and Falcone A (2016). Location of Primary Tumor and Benefit From Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies in Patients With RAS and BRAF Wild-Type Metastatic Colorectal Cancer. Oncologist 21(8): 988-994.
Pietrantonio F, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, Cabiddu M, Iacovelli R, Bossi I, Lonati V, Ghilardi M, de Braud F and Barni S (2015). Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. Eur J Cancer 51(5): 587-594.
Price T, Kim TW, Li J, Cascinu S, Ruff P, Suresh AS, Thomas A, Tjulandin S, Guan X and Peeters M (2016). Final results and outcomes by prior bevacizumab exposure, skin toxicity, and hypomagnesaemia from ASPECCT: randomized phase 3 non-inferiority study of panitumumab versus cetuximab in chemorefractory wild-type KRAS exon 2 metastatic colorectal cancer. Eur J Cancer 68: 51-59.
R Core Team (2015). A language and environment for statistical computing., R Foundation for Statistical Computing.
Rankin A, Klempner SJ, Erlich R, Sun JX, Grothey A, Fakih M, George TJ, Jr., Lee J, Ross JS, Stephens PJ, Miller VA, Ali SM and Schrock AB (2016). Broad Detection of Alterations Predicted to Confer Lack of

Benefit From EGFR Antibodies or Sensitivity to Targeted Therapy in Advanced Colorectal Cancer. Oncologist.
Rowland A, Dias MM, Wiese MD, Kichenadasse G, McKinnon RA, Karapetis CS and Sorich MJ (2015). Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. Br J Cancer 112(12): 1888-1894.
Sartore-Bianchi A, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S, Di Nicolantonio F, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S and Bardelli A (2009). PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 69(5): 1851-1857.
Seligmann JF, Elliott F, Richman SD, Jacobs B, Hemmings G, Brown S, Barrett JH, Tejpar S, Quirke P and Seymour MT (2016). Combined Epiregulin and Amphiregulin Expression Levels as a Predictive Biomarker for Panitumumab Therapy Benefit or Lack of Benefit in Patients With RAS Wild-Type Advanced Colorectal Cancer. JAMA Oncol.
Song N, Liu S, Zhang J, Liu J, Xu L, Liu Y and Qu X (2014). Cetuximab-induced MET activation acts as a novel resistance mechanism in colon cancer cells. Int J Mol Sci. 15(4): 5838-5851. doi: 5810.3390/ijms15045838.
Tejpar S, Stintzing S, Ciardiello F, Tabernero J, Van Cutsem E, Beier F, Esser R, Lenz HJ and Heinemann V (2016). Prognostic and Predictive Relevance of Primary Tumor Location in Patients With RAS Wild-Type Metastatic Colorectal Cancer: Retrospective Analyses of the CRYSTAL and FIRE-3 Trials. JAMA Oncol.
Temraz S, Mukherji D and Shamseddine A (2016). Dual targeting of HER3 and EGFR in colorectal tumors might overcome anti-EGFR resistance. Crit Rev Oncol Hematol. 101:151-7.(doi): 10.1016/j.critrevonc.2016.1003.1009. Epub 2016 Mar 1010.
Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC and Gwyther SG (2000). New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92(3): 205-216.
Troiani T, Martinelli E, Napolitano S, Vitagliano D, Ciuffreda LP, Costantino S, Morgillo F, Capasso A, Sforza V, Nappi A, De Palma R, D'Aiuto E, Berrino L, Bianco R and Ciardiello F (2013). Increased TGF-alpha as a mechanism of acquired resistance to the anti-EGFR inhibitor cetuximab through EGFR-MET interaction and activation of MET signaling in colon cancer cells. Clin Cancer Res. 19(24): 6751-6765. doi: 6710.1158/1078-0432.CCR-6713-0423. Epub 2013 Oct 6711.
Troncale S, Barbet A, Coulibaly L, Henry E, He B, Barillot E, Dubois T, Hupe P and de Koning L (2012). NormaCurve: a SuperCurve-based method that simultaneously quantifies and normalizes reverse phase protein array data. PLoS One. 7(6): e38686. Epub 32012 Jun 38628.
Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pinter T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J and Rougier P (2009). Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med. 360(14): 1408-1417. doi: 1410.1056/NEJMoa0805019.
Van Emburgh BO, Sartore-Bianchi A, Di Nicolantonio F, Siena S and Bardelli A (2014). Acquired resistance to EGFR-targeted therapies in colorectal cancer. Mol Oncol 14(14): 00096-00099.
Van Schaeybroeck S, Karaiskou-McCaul A, Kelly D, Longley D, Galligan L, Van Cutsem E and Johnston P (2005). Epidermal growth factor receptor activity determines response of colorectal cancer cells to gefitinib alone and in combination with chemotherapy. Clin Cancer Res. 11(20): 7480-7489.
Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Ercan D, Rogers A, Roncalli M, Takeda M, Fujisaka Y, Philips J, Shimizu T, Maenishi O, Cho Y, Sun J, Destro A, Taira K, Takeda K, Okabe T, Swanson J, Itoh H, Takada M, Lifshits E, Okuno K, Engelman JA, Shivdasani RA, Nishio K, Fukuoka M,

Varella-Garcia M, Nakagawa K and Janne PA (2011). Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med 3(99): 99ra86.

**Table 1: Description of Clinical Variables of mCRC samples**

| | | N | % |
|---|---|---|---|
| Sex | Man | 20 | 58.8 |
| | Female | 14 | 41.4 |
| Age at diagnosis | < 50 | 11 | 32.4 |
| | ≥ 50 | 23 | 67.6 |
| Localization | 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 |
| | Stabilization | 11 | 32,4 |
| | Progression | 12 | 35,3 |
| Line of treatment | 1st line | 4 | 11,8 |
| | 2nd line | 13 | 38,2 |
| | 3rd 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 |

**Titles and Legends to Figure**

**Figure 1:** Differences in pathway activation between left-sided versus right-sided CRC. **A**. Phosphorylated proteins that are differentially expressed ($p<0.05$) between left-sided and right-sided CRC were analyzed using Ingenuity Pathway Analysis and found to be enriched in ErbB and Wnt signaling. Enrichment is calculated against the list of analyzed proteins. The indicated threshold of -log(p-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 color gradient of the proteins represents the fold change between left- and right-sided CRC, with a darker color indicating a greater fold-change. White proteins are part of the pathway but have not been analyzed 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 (STA) + progressive disease (Pro) versus partial response (RP) . No complete response (CR) 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.

**Figure 3**: 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.

**Figure 4:** Simplified scheme of signaling interaction network between the proteins that are associated with response to anti-EGFR therapy. The red color gradient reflects the p-value with a darker color

indicating a lower p-value. Only direct and experimentally proven interactions between proteins, described in literature, are shown.

**Supplementary data**

**Supplementary Figure 1**: Hierarchical clustering of the RPPA data shows two distinct groups of samples that are not related to response to therapy (color bar 1), to whether the tumor is right- or left-sided (color bar 2), to the center of origin (color bar 3) or to the type of tumor (color bar 4). Samples are horizontal, antibodies are vertical. White: missing data.

**Supplementary Figure 2**: Kaplan Meier curves of overall survival in right- and left-sided mCRC.

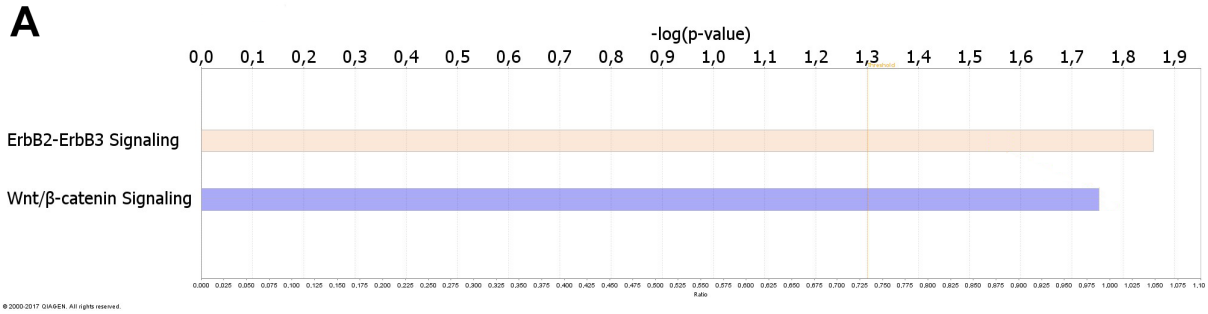
**Supplementary figure 3**: PI3K pathway activation status in *PIK3CA* wildtype (WT) and mutant (MUT) samples, showing the ratios of Phospho-Akt / Akt (p=0.11), phospho-p70S6K /p70S6K (p=0.14) and phospho-PKCα/ PKCα (p=0.10).

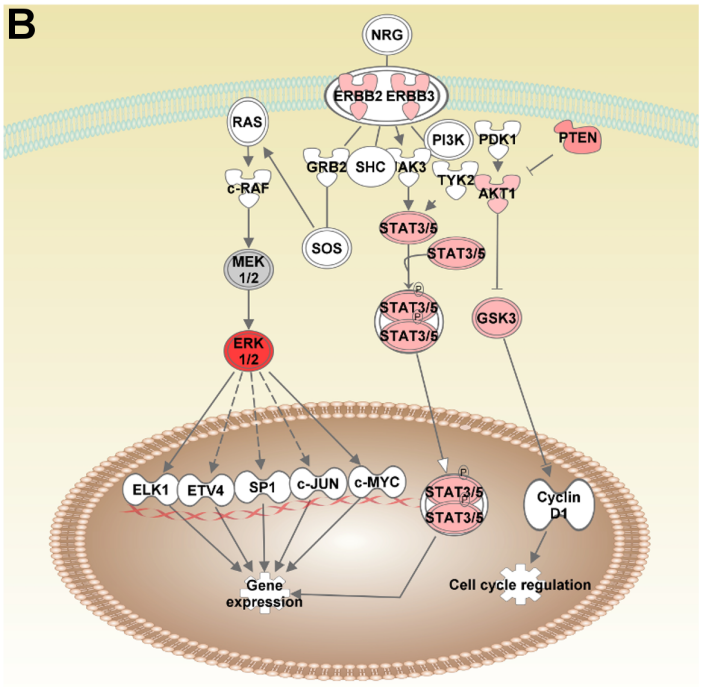
**Supplementary Table 1: Antibodies used for RPPA analyses**
**R: Rabbit, M: Mouse, CST: Cell Signaling Technology, BD: BD Biosciences**

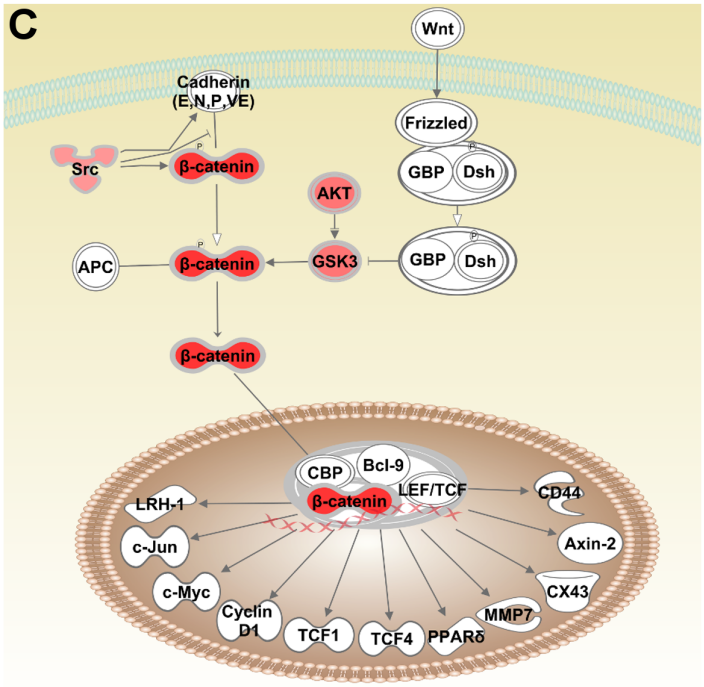
| Name | Species | Supplier | Reference |
|---|---|---|---|
| **Tyrosine kinases / receptors** | | | |
| **EGFR (D38B1)** | R | CST | 4267 |
| **Phospho-EGFR (Tyr 992)** | R | CST | 2235 |
| **Phospho-EGFR (Thr669)** | R | CST | 3056 |
| **Phospho-EGFR (Tyr 1173) (53A5)** | R | CST | 4407 |
| **HER2/ErbB2** | M | Thermo | MA5-14057 |
| **HER2/ErbB2** | M | Lab Vision | MS-1350-P1 (Ab20) |
| **Phospho-HER2/ErbB2 (Tyr1139)** | R | Epitomics | 1991-1; ab53290 |
| **FGF Receptor3 (D2G7E)** | R | CST | 3163 |
| **FGF receptor4** | R | CST | 8562 |
| **HER3/ErbB3 (c-17)** | R | Santa-Cruz | sc-285 |
| **Phospho-Her3/Erbb3 (tyr1289)** | R | CST | 4791 |
| **HER4/ErbB4** | R | Epitomics | 2218-1 |
| **Phospho-HER4 (Tyr1162)** | R | Epitomics | 2295-1 |
| **FAK** | R | CST | 3285 |
| **Phospho-FAK (Tyr861)** | R | Epitomics | 2153-1 |
| **Src (36D10)** | R | CST | 2109 |
| **Phospho-Src (Tyr527)** | R | CST | 2105 |
| **Met** | R | Santa-Cruz | sc-10 (C-12) |
| **Phospho-Met (Tyr1349)** | R | CST | 3133 |
| **IGF-I receptor B** | R | CST | 9750 |
| | | | |
| **PI3K/Akt pathway** | | | |
| **PI3 Kinase p110 subunit Beta** | R | CST | 3011 |
| **Akt** | R | CST | 9272 |
| **Phospho-Akt (Thr308) (D25E6)** | R | CST | 13038 |
| **Phospho-Akt (Ser473) (193H12)** | R | CST | 4058 |
| **PTEN (D4.3) XP** | R | CST | 9188S |
| **Phospho-PTEN (ser380/Thr382/383)** | R | CST | 9554 |
| **mTOR** | R | Abcam | ab51089 |
| **phospho-mTOR (Ser2448)** | R | Abcam | ab109268 |
| **p70 S6 Kinase** | R | CST | 2708 |
| **Phospho-p70 S6 kinase (Thr421/Ser424)** | R | Upstate (Millipore) | 04-393 |
| **Phospho-p70 S6 Kinase (Thr389)** | R | CST | 9205 |
| **S6 Ribosomal Protein (5G10)** | R | CST | 2217 |
| **Phospho-S6 Ribosomal Protein (Ser235/236)** | R | CST | 2211 |

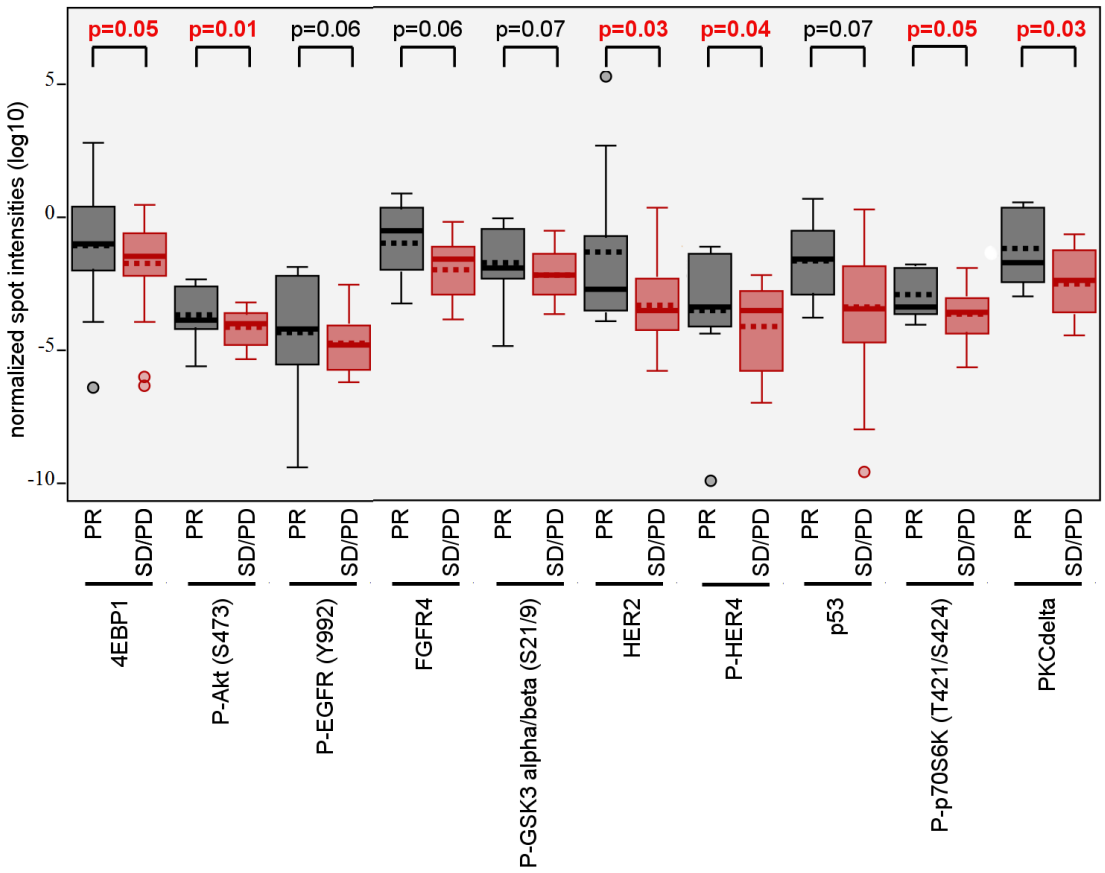
| | | | |
|---|---|---|---|
| **Ras/MAPK pathway** | | | |
| **K-RAS** | M | Santa-Cruz | sc-30 (F234) |
| **B-Raf** | R | Santa-Cruz | sc-166 |
| **MEK1/2** | R | CST | 9122S |
| **Phospho-MEK1/2 (Ser217/221)** | R | CST | 9154 |
| **p44/42 MAPK** | R | CST | 9102 |
| **Phospho-p44/42 MAPK (Thr202/Tyr204)** | R | CST | 4377 (197G2) |
| | | | |
| **Downstream effectors** | | | |
| **4E-BP1** | R | CST | 9452 |
| **Phospho-4E-BP1 (Thr70)** | R | Epitomics | 2250-1 |
| **eIF4E** | R | CST | 9742 |
| **eIF4B** | R | CST | 3592 |
| **Phospho-eIF4B (Ser422)** | R | CST | 3591 |
| **PLC gamma1** | R | CST | 2822 |
| **Phospho-PLC gamma1 (Tyr771)** | R | Epitomics | 2350-1 |
| **Phospho-PLC gamma1 (Tyr783)** | R | CST | 2821 |
| | | | |
| **Proliferation/Apoptosis** | | | |
| **p53** | R | CST | 9282 |
| **Phospho-p53 (Ser392)** | R | Epitomics | 2326-1 |
| **Phospho-p53 (Ser15)** | R | CST | 9284 |
| **Caspase7** | R | Epitomics | 1032-1 |
| **Cleaved Caspase7 (Asp198)** | R | CST | 9491 |
| **Cleaved Caspase8 (Asp391)** | R | Thermo | MA5-15054 |
| **PARP uncleaved p116** | R | Epitomics | 1077-1 / ab32378 |
| **Cleaved PARP (Asp214) p25** | R | Epitomics | 1051-1 |
| **BAD** | M | BD | 610391 |
| **Bcl2** | R | CST | 2876 |
| **Cyclin D1** | R | Epitomics | 2261-1 |
| **Topoisomerase II alpha** | R | Epitomics | 1826-1 |
| **Phospho-Topoisomerase II a (Thr1343)** | R | Epitomics | 1871-1 / ab52853 |
| **Ki67 (MIB-1)** | M | Dako | M7240 |
| **Cytochrome c (136F3)** | R | CST | 4280 |
| | | | |
| **Angiogenesis** | | | |
| **VEGF Receptor1 (clone Y103)** | R | Novus | NB110-57643 |
| **Phospho-VEGF Receptor2 (Tyr1175)** | R | Novus | NB100-82260 |
| **PDGFR beta** | R | CST | 3169 |

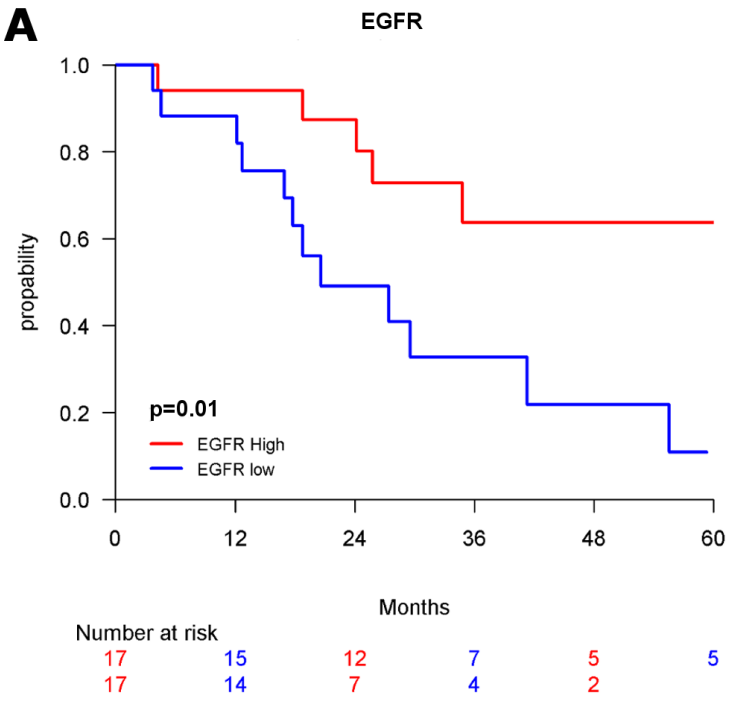
| | | | |
|---|---|---|---|
| **phospho-PDGFR beta (Tyr1021)** | R | CST | 2227 |
| | | | |
| **Jak/Stat** | | | |
| **Stat1** | R | CST | 9172 |
| **Phospho-Stat1 (Y701)** | R | Abcam | ab109457 |
| **Stat3** | R | CST | 9132 |
| **Phospho-Stat3 (Tyr705) (D3A7)** | R | CST | 9145 |
| **Phospho-Stat3 (Ser727)** | R | CST | 9134 |
| **Jak2 (D2E12)** | R | CST | 3230 |
| | | | |
| **Protein Kinase C** | | | |
| **PKC alpha** | M | Upstate (Millipore) | 05-154 |
| **Phospho-PKC alpha (Ser657)** | R | Upstate (Millipore) | 06-822 |
| **PKC delta** | R | Epitomics | 2053-1 |
| **Phospho-PKC delta (Thr505)** | R | CST | 9374 |
| | | | |
| **TGF beta** | | | |
| **TGF-beta I/III (56E4)** | R | CST | 3709 |
| **Smad3 (P84022)** | R | Epitomics | 1735-1 |
| **Phospho-Smad3 (Ser423/425)** | R | Epitomics | 1880-1 |
| | | | |
| **Wnt/NOTCH** | | | |
| **Beta Catenin (6B3)** | R | CST | 9582 |
| **Phospho-Beta Catenin (Ser675)** | R | CST | 4176 |
| **Notch1** | R | Epitomics | 1935-1 |
| **Cleaved Notch1 (Val1744) (D3B8)** | R | CST | 4147 |
| **GSK3 alpha/beta (0011-A)** | M | Santa-Cruz | sc-7291 |
| **Phospho-GSK3 alpha/beta (Ser21/9)** | R | CST | 9331 |
| **Phospho-GSK3 alpha/beta (Tyr279/216)** | R | Epitomics | 2309-1 |

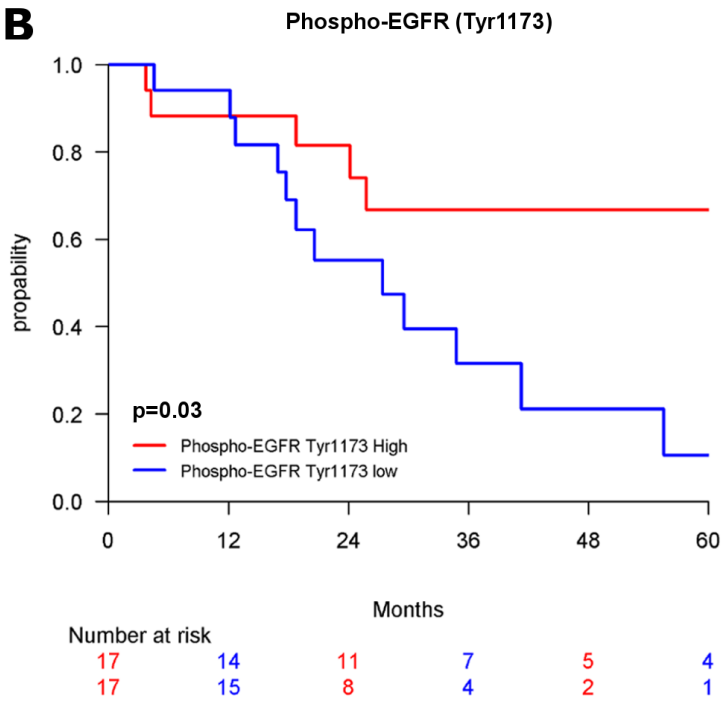

A
-log(p-value)
0,0 0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1,0 1,1 1,2 1,3 1,4 1,5 1,6 1,7 1,8 1,9
ErbB2-ErbB3 Signaling
Wnt/β-catenin Signaling
Ratio
© 2000-2017 QIAGEN. All rights reserved.

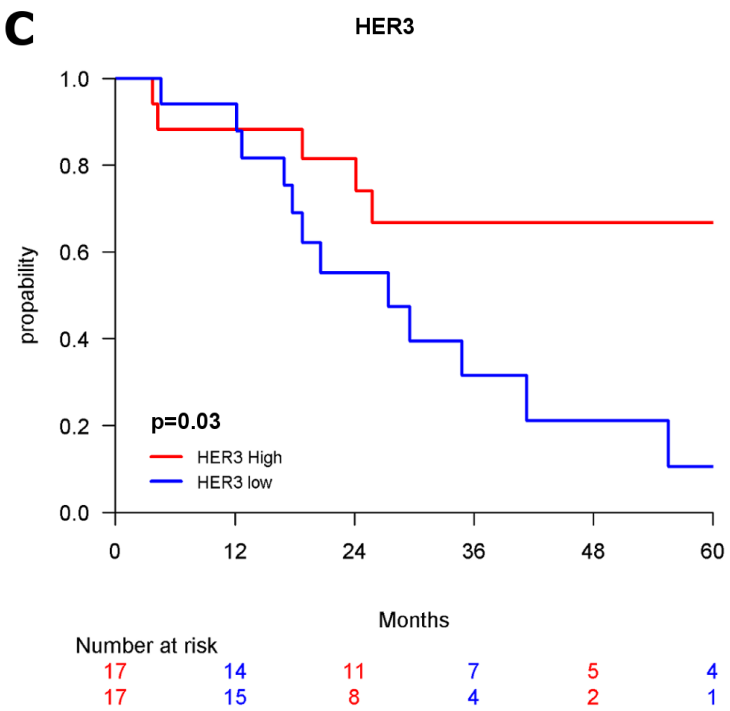

B
NRG
ERBB2 ERBB3
RAS
PI3K
PDK1
PTEN
GRB2
SHC
JAK3
TYK2
AKT1
c-RAF
STAT3/5
SOS
STAT3/5
MEK 1/2
STAT3/5
STAT3/5
GSK3
ERK 1/2
ELK1
ETV4
SP1
c-JUN
c-MYC
STAT3/5
STAT3/5
Cyclin D1
Gene expression
Cell cycle regulation

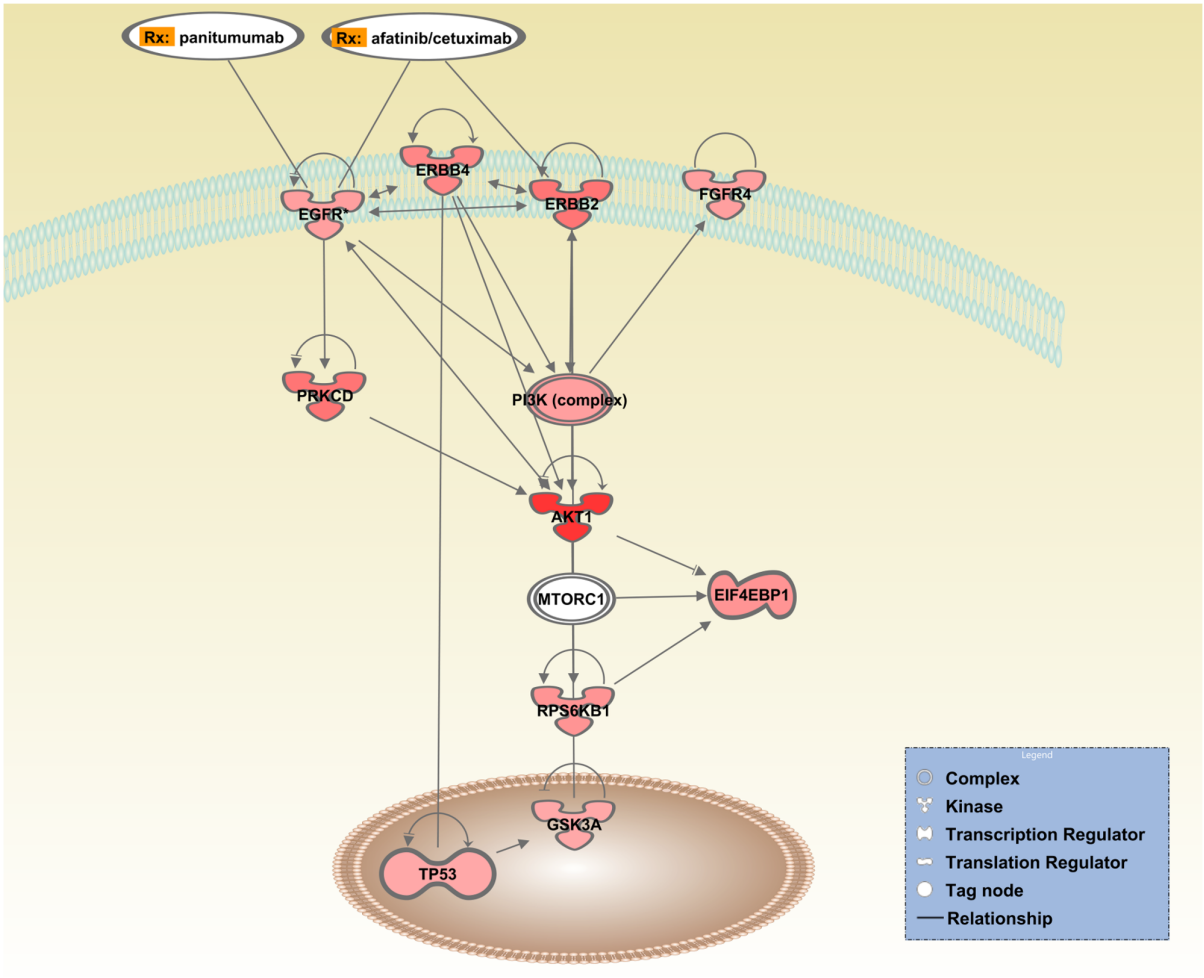

C
Wnt
Cadherin (E,N,P,VE)
Frizzled
Src
β-catenin
GBP
Dsh
AKT
APC
β-catenin
GSK3
GBP
Dsh
β-catenin
CBP
Bcl-9
LRH-1
β-catenin
LEF/TCF
CD44
c-Jun
Axin-2
c-Myc
CX43
Cyclin D1
TCF1
TCF4
PPARδ
MMP7


p=0.05
p=0.01
p=0.06
p=0.06
p=0.07
p=0.03
p=0.04
p=0.07
p=0.05
p=0.03
normalized spot intensities (log10)
5
0
-5
-10
PR
SD/PD
4EBP1
P-Akt (S473)
P-EGFR (Y992)
FGFR4
P-GSK3 alpha/beta (S21/9)
HER2
P-HER4
p53
P-p70S6K (T421/S424)
PKCdelta


A
EGFR
1.0
0.8
0.6
0.4
0.2
0.0
propability
p=0.01
EGFR High
EGFR low
0
12
24
36
48
60
Months
Number at risk
17 15 12 7 5 5
17 14 7 4 2


B
Phospho-EGFR (Tyr1173)
1.0
0.8
0.6
0.4
0.2
0.0
propability
p=0.03
Phospho-EGFR Tyr1173 High
Phospho-EGFR Tyr1173 low
0
12
24
36
48
60
Months
Number at risk
17 14 11 7 5 4
17 15 8 4 2 1


C
HER3
1.0
0.8
0.6
0.4
0.2
0.0
propability
p=0.03
HER3 High
HER3 low
0
12
24
36
48
60
Months
Number at risk
17 14 11 7 5 4
17 15 8 4 2 1


Rx: panitumumab
Rx: afatinib/cetuximab
ERBB4
EGFR*
ERBB2
FGFR4
PRKCD
PI3K (complex)
AKT1
MTORC1
EIF4EBP1
RPS6KB1
GSK3A
TP53
Legend
Complex
Kinase
Transcription Regulator
Translation Regulator
Tag node
Relationship