TESI DOCTORAL UPF / 2012

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Molecular Therapeutics and Biomarkers in Breast Cancer

Cancer Research Program

Hospital del Mar Research Institute (IMIM)

Abans de res, vull de donar les gràcies a totes aquelles persones que m'han ajudat i amb qui he compartit el transcurs d'aquesta tesis:

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ABSTRACT

EGFR is a transmembrane tyrosine kinase receptor from the HER family which, upon stimulation by its specific ligands, forms homo- or hetero- dimers with other HER family members leading to the activation of different signaling pathways which are key mechanisms in tumorogenesis, involving cell proliferation and survival. EGFR can be targeted by monoclonal antibodies, as cetuximab and panitumumab, which bind to EGFR with high affinity preventing ligand binding and stimulation of the receptor.

The approval of cetuximab and panitumumab represents a milestone in the treatment of colorectal cancer. However, its clinical success is uniformily limited by the development of acquired drug resistance.

We describe a new mechanism of acquired resistance to cetuximab in colorectal cancer that was due to a missense mutation in the EGFR ectodomain (S492R mutation). Upon chronic exposure to cetuximab, colorectal cancer cell lines acquired S492R mutation and became resistant to the treatment. We observed that cetuximab was not able to bind mutant EGFR. Notably, this amino acid change did not affect the ability of panitumumab to bind to EGFR, and panitumumab effectively suppressed growth of mutant cells. EGFRS492R mutation was detected in 2 out of 10 tumor specimens from patients following progression on cetuximab. One of these patients was subsequently treated with single agent panitumumab yielding a partial response. The S492R mutation defines a novel biomarker of resistance to cetuximab but not to panitumumab in colorectal cancer. Although both antibodies are generally considered as equivalent therapies and oncologist are not currently inclined to treat patients with

panitumumab following cetuximab treatment failure, our results suggest that both antibodies might have different effects in tumor cells and testing the presence of EGFR extracellular domain mutation after cetuximab failure may be predictive of benefit associated with subsequent panitumumab therapy.

RESUM

EGFR és un receptor transmembrana tirosina cinasa de la família HER el qual, després de l'estimulació mitjançant lligands específics, homo o heterodimeritza amb els altres membres de la família HER donant lloc a l'activació de diferents vies de senyalització cel·lular que són mecanismes claus en processos tumorogènics com proliferació i supervivència cel·lular. L'EGFR es pot inhibir amb anticossos monoclonals, com el cetuximab i el panitumumab, que s'uneixen al receptor amb molta afinitat prevenint la unió i activació del receptor per part dels lligands.

L'aprovació de cetuximab i panitumumab representen un gran avenç en el tractament del càncer colorectal, però el seu ús sempre es veu limitat per el desenvolupament de resistència adquirida al tractament.

Nosaltres describim un mecanisme de resistència adquirida a cetuximab enl càncer colorectal degut a l'adquisió d'una mutació en el domini extracel·lular de l'EGFR, la mutació S492R. Al llarg de l'exposició crònica a cetuximab, linies cel·lulars de càncer colorectal van adquirir la mutació S492R i es van tornar resistents al tractament. Vam observar que cetuximab no era capaç d'unir-se a l'EGFR mutat. Aquests canvi d'aminoàcid no afectava a l'habilitat que té panitumumab a unir-se al EGFR, pertant, panitumumab és capaç de suprimir el creixement de les cel·lules tumorals mutades. Vam detectar la mutació EGFRS492R en 2 de 10 mostres tumorals de pacients que havien recaigut al tractament amb cetuximab. Un d'aquest pacients va ser posteriorment tractat amb panitumumab i va obtenir una resposta tumoral parcial.

La mutació S492R defineix un nou mecanisme de resistència a cetuximab però no a panitumumab en el tractament del càncer colorectal. Malgrat que aquest anticossos generalment es consideren com teràpies equivalents i els oncòlegs no solen tractar els pacients de càncer colorectal amb panitumumab un cop han recaigut al tractament amb cetuximab, els nostres resultats suggereixen que els dos anticossos poden tenir efectes diferents en les cèl·lules tumorals i que la determinació la mutació del domini extracel·lular de l'EGFR després de recaure a cetuximab podria preveure el benefici associat a una posterior teràpia amb panitumumab.

PROLOGUE

The work presented in this PhD Thesis has been conducted in the Molecular Therapautics and Biomarkers in Breast Cancer laboratory from the IMIM Cancer Research Program. The group, leaded by Dr. Joan Albanell, is divided in two different arees; the preclinical laboratory, coordinated by Dr. Ana Rovira and the translational laboratory coordinated by Dr. Federico Rojo. Both laboratories together comprise a multidisciplinary team composed by oncologists, pathologists and biologists and joint the knowledge of the different disciplins to work in translational projects with clinical relevant interest.

From September 2007 since April 2012, I have worked and learned from different research projects. During the first two years, I collaborated in several studies in breast cancer that have already been published in different scientific journals. From 2009 until now, I have been working in a FIS project leaded by Dr. Clara Montagut. The results presented in this PhD thesis are focused on this project and were published in January 2012. The work was done in close collaboration with the Oncology and Pathology department of Hospital del Mar, and with key collaborators from the Center for Genomic Regulation, the Hemathology department of Vall d'Hebrón University Hospital and the Discovery Oncology department of Genentech in San Francisco, USA.

Although different therapeutic antibodies are approved for cancer treatment, little is known about how tumors develop resistance to them. The work of this PhD Thesis has led to the identification of a molecular mechanism of resistance to cetuximab, a therapeutic

antibody, in colorectal cancer. The translational model of our group has been key for this PhD thesis; by joining efforts from clinicians and basic researchers, we have provided by preclinical tools an answer to a question made from the clinical practice, with the ultimate goal of improving personalized cancer medicine.

INDEX

ABREVIATIONS AND ACRONYMS

%: Percentage

ADCC: Antibody Dependent Cell-mediated Cytotoxicity

ATP: Adenosine Triphosphate

CRC: Colorectal Cancer

mCRC: metastatic Colorectal Cancer

CT: Computed Tomography

ES: Embryonic Stem cells

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ERK: Extracellular Regulated Kinase

FFPE: Formalin-Fixed Paraffin Embedded

FISH: Fluorescence *in situ* Hybridization

FOLFOX: Folinic Acid, 5-Fluorouracil, Oxaliplatin

FOLFIRI: Folinic Acid, 5-Fluorouracil, Irinotecan

GEF: Guanine Exchange Factor

GDP: Guanosine DiPhosphate

GTP: Guanosine TriPhosphate

HB-EGF: Heparine Bind Epidermal Growth Factor

HGF: Hepatocyte Growth Factor

HNSCC: Head and Neck Squamous Cell Carcinoma

IHC: Immunohistochemistry

IGFR: Insulin-like Growth Factor Receptor

JAK: Janus Kinase

MAPK: Mitogen Activated Protein Kinase

MAPKK: Mitogen Activated Protein Kinase Kinase

MAPKKK: Mitogen Activated Protein Kinase Kinase Kinase

MEF: Mouse Embryonic Fibroblast

MMP: Matrix Metalloproteinase

mTOR: mammalian Target of Rapamycin

moAb: monoclonal Antibody

mRNA: Messenger Ribonucleic Acid

NRG: Neuregulin

NSCLC: Non-Small Cell Lung Cancer

PCR: Polimerase Chain Reaction

PDK1: 3-Phosphoinositide dependent protein Kinase-1

PI3K: Phosphoinositide 3-kinase

PiP_2: Phosphatidylinositol-4,5-biPhosphate

PiP_3: Phosphatidylinositol-3,4,5-triPhosphate

PTB: Phosphotyrosine Binding

PTEN: Phosphatase and Tensin homolog

RTK: Receptor Tyrosine Kinases

Ser: Serine

SH2: Src Homology domain

STAT: Signal Transducer and Activator of Transcription

TGFα: Transforming Growth Factor alpha

Thr: Threonine

TK: Tirosine Kinase

Tyr: Tyrosine

VEFG: Vascular Endothelial Growth Factor

WB: Western Blot

WT: Wild-Type

INTRODUCTION

I.1. COLORECTAL CANCER OVERVIEW

I.1.1. Importance of colorectal cancer

Colorectal cancer is the first leading cause of death by cancer in Europe and the fourth world wide, accounting for 8% of all cancer deaths. As observed for incidence, mortality rates are lower in women than in men and almost 60% of the cases occur in developed regions[1,2].

Due to its high incidence and mortality, a lot of work has been done in the last years to understand the molecular events underlying this disease and to develop new therapeutic strategies, requiring the effort to join the knowledge of basic researchers, clinicians and the pharmaceutical industry. Although life expectancy has increased with novel treatments, unresectable metastatic colorectal cancer remains incurable and many questions need to be solved to improve the management of this disease.

I.1.2. Biology of colorectal cancer

Normal intestinal epithelium is continuously renewing while maintaining a precise balance between proliferation, differentiation, cell migration and cell death. Intestinal tumorogenesis occurs when the mechanisms that control these processes are altered, thus leading to altered tissue homeostasis (reviewed in[3]). CRC is initiated in the colon as an epithelial hyperplasia that becomes increasingly dysplastic resulting in aberrant crypt formation[4]. This process progress from

benign tumors, termed adenomas, to malignant tumors termed carcinomas. The accessibility of the lesions and the fact that different stages of the disease coexist have allowed the study of the genetic alterations present at the different stages of the malignancy[5].

In 1990 it was proposed a model which suggested that the adenoma-carcinoma-metastasis transition occurs by the accumulation of specific genetic alterations. First, colorectal tumors appear to arise as a result of the mutational activation of oncogenes and the mutational inactivation of tumor suppressor genes. Second, mutations in at least four to five genes are required for the formation of malignant tumors. Third, although the genetic alterations often occur according to a preferred sequence, the total accumulation of changes is the responsible for determining tumor's biologic properties[5]. (**Figure I.1**).

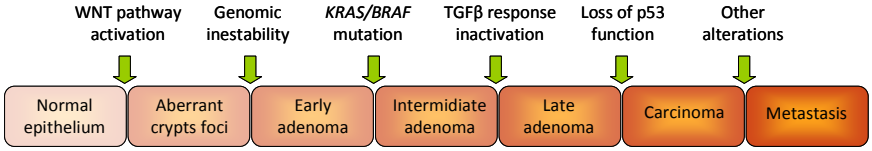


Figure I.1. Genetic model of colorectal carcinogenesis (adapted from Fearon and Vogelstein[5]). Tumor initiation requires aberrant activation of WNT pathway. Genomic instability and subsequent acquisition of sequential mutations lead to the progression from adenoma to metastatic carcinoma.

Tumor initiation requires aberrant activation of the WNT pathway. (Reviewed in[6,7]) Furthermore, pathways which regulate genomic stability are altered and lead to the accumulation of the specific mutations to acquire the "genetic signature" (**Figure I.1**) responsible for the evolution from the adenoma to the malignant carcinoma

(Reviewed in[8]). There is a complex interaction among these pathways and all of them are found, at least in some degree, in all CRC[7].

I.1.3 Colorectal cancer in the clinical practice

Once a patient has been diagnosed for CRC its prognosis is clearly related to the stage of the disease at the moment of diagnosis[9]. In Stages 0, I, II, the lesion is confined to the primary tumor and there is neither lymph nodes involvement nor distant metastasis, the treatment is limited to local surgery and anastomosis if necessary[10].

In Stage III, where there is lymph nodes involvement, it has been demonstrated that adjuvant chemotherapy after surgery reduces the risk of relapse and increases disease free survival[11].

In Stage IV the disease has disseminated to distant sites (i.e. metastasis) and therefore the prognosis is worse and the goal of the treatment is to prolong survival and maintain quality of live for as long as possible[12]. However, a particular subset of patients at this stage, those whose metastasis are confined to the liver, can be curable by surgical resection of the metastatic lesions[13,14]

Despite the bad prognosis of mCRC, the last decade has seen significant advances in its treatment (Reviewed in [15]). Where we once had only a single agent available for treatment and median overall survival was no more than 15 months, now there are 7 pharmaceutical agents active and approved for the treatment of this disease and life expectancy is more than 2 years (**Figure I.2**).

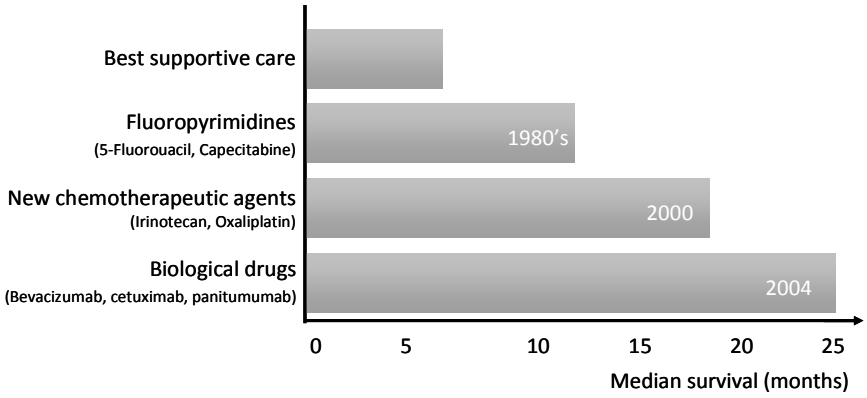


Figure I.2. Life expectancy of mCRC patients has increased by the addition of new treatments. The graph shows the increase in median survival of patients related to the treatments that have been approved for mCRC since the early 80's.

The agents approved for mCRC treatment include 5-Fluorouracil, Capecitabine, Irinotecan and Oxaliplatin as chemotherapeutic agents and Bevacizumab[16](anti-VEGF), Cetuximab[17] (anti-EGFR moAb) and Panitumumab[18] (anti-EGFR moAb) as targeted therapies.

Studies providing information regarding differences between Combination vs. Sequential treatment[19,20] have also been done. Even overall survival was found to be similar by both therapeutic approaches, combined regimen lead to a longer Progression Free Survival and better response rates. Therefore, combination chemotherapy is the choice for those patients who may become eligible for curative liver resection following chemotherapy to shrink tumor size. And sequential treatments are preferred in some settings when the goal is to prolong life expectancy with minimum side effects.

The role of Biological agents, particularly anti-EGFR moAbs, will be discussed in the following sections.

I.2. EGFR PATHWAY

EGFR, also known as HER1 or ErbB1, is a typical RTK that was implicated in cancer in the early 1980's, when it was found to be aberrantly encoded by the virus of the avian eritroblastosis tumor[21]. Since then, the EGFR family, also known as HER or ErbB family, of receptors has increased to four different members.

The EGFR signaling pathway is a classical transduction pathway where a ligand binds to a monomeric receptor activating its catalytic function by promoting receptor dimerization and autophosphorylation on cytoplasmic tyrosine residues. These residues serve as docking sites for adaptor proteins or enzymes, which simultaneously initiate several signaling cascades to produce a physiological outcome. Due to the large number of ligands, receptors and adaptor proteins that are involved in this pathway, it has been related with many different cellular functions as cell division, migration, cell adhesion, differentiation and also apoptosis. This rich multilayered network permits context-specific responses throughout development and adulthood (reviewed in [22]).

I.2.1. The HER family

The HER family includes for receptor: EGFR/ErbB1, ErbB2, ErbB3 and ErbB4 (also known as Her1, Her2, Her3 and Her4) which are expressed in tissues from epithelial, mesenchymal and neuronal origin [23-26]. As all RTKs, each member comprises an extracellular ligand-binding region, a single membrane-spanning region, a cytoplasmic

TK-containing domain and a C-terminal regulatory region (reviewed in[27]).

Under physiological conditions, activation of HER receptors is controlled by the spatial and temporal expression of their ligands, which are members of the EGF family of growth factors. Ligand binding to the receptor induces the formation of homo and heterodimers resulting in the activation of the TK domain, which phosphorylates specific tyrosine residues within the cytoplasmatic tail (reviewed in [22]).

Despite the similarity between all HERs, several differences among their structure and ligand binding affinity exists. HER2 does not have any known ligand[28]; however, it is the preferred partner for heterodimerization with other members of the family. HER3 is devoid of intrinsic kinase activity so heterodimeryzation of this receptor is crucial for its activity[29,30]. Moreover, HER ligands have different affinities among the receptors; while EGF, TGFα, and amphiregulin are specific for EGFR binding, β-cellulin, HB-EGF and epiregulin show dual specificity binding both EGFR and HER4. Another group of ligands are the neuregulins (NRG), from which NRG1 and NRG2 bind both HER3 and HER4, and NRG3 and NRG4 are only able to bind ErbB4 (reviewed in [31]). These differences among HER family members appear to explain why a horizontal network of interactions is crucial to the HER signaling.

I.2.2. EGFR structure and its mechanism of activation

Although EGFR high-resolution structural studies with the intact receptor have not yet been done, there is a wealth of structural data on

both extra and intracellular regions in unstimulated and stimulated states.

EGFR gene is localized in chromosome 7 and codifies for a 1210-residue polypeptide precursor which, after being synthesized, its signal peptide is cleaved leading to a 1186-residue protein that is glycosylated and inserted into the cell membrane[32]. Residues 24 to 645 code for the extracellular region of EGFR which contains for different domains; domain I and domain III are homologous ligand binding domains (also referred as L1 and L2), while domain II and domain IV are two cystine rich domain (also referred as CR1 and CR2)[33,34]. The transmembrane and juxtamembrane domain is an α-helix codified by residues 646 to 712 and it appears to have a number of regulatory functions as ligand independent internalization events and basolateral sorting of EGFR in polarized cells[35-37]. The TK domain comprises residues 713 to 979 and is similar to other tyrosine kinases, where the ATP sits between the N-terminal lobe dominated by a β-sheet and a larger C-terminal lobe mainly α-helix[38]. Finally, the carboxy-terminal domain of EGFR contains tyrosine and serine/threonine residues whose phosphorylation modulates EGFR-signal transduction pathways[38].

In unstimulated conditions, most of EGFR molecules exhibit an autoinhibited or tethered conformation, in which domain II and IV form an intramolecular interaction hiding a loop in domain II (dimerization arm) that prevents the interaction with other receptors. The remaining 5% of EGFR molecules adopt a range of untethered conformations to different extents (**Figure I.3.**). In the presence of the ligands, they preferentially bind to unthetered molecules interacting simultaneously with domain I and domain III stabilizing

this particular conformation in which the dimerization arm in domain II is exposed and the receptor can dimerize with another ligand bound receptor[39,40].

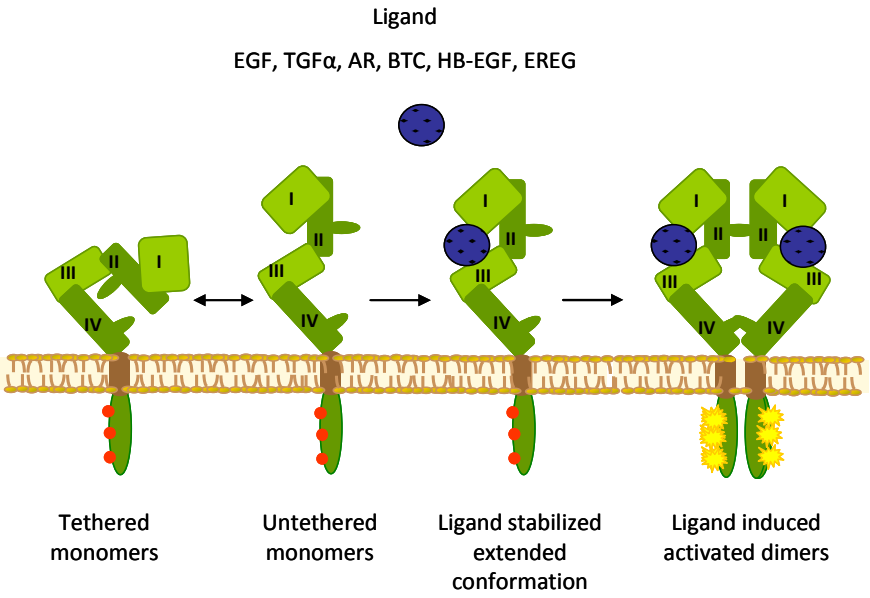


Figure I.3. Mechanism of ligand-induced EGFR dimerization. EGFR structure within the cell membrane is represented in the figure; EGFR ECD, which is comprised by 4 different domains (I, II, III, IV in green) , transmembrane domain (in brown) and intracellular domain with tyrosine residues (red dots). About 95% of EGFR exist in a compact autoinhibited or tethered conformation, in the remaining 5% of the unligated molecules EGFR ECD adopt a range of untethered conformations. Ligand binds to untethered molecules interacting simultaneously with domains I and III and stabilizes the particular extended form in which domain II is exposed and the receptor can dimerize. When two receptors dimerize, the intracellular domains approach and TK activity is enhanced phosphorylating the tyrosine residues of both receptors (yellow marks). Figure adapted from[27,41]

Once the receptor dimer is formed, the TK domain adopts a conformation in which is activated and phosphorylates the subsequent residues of the C-terminal domain, these phosphorylated residues recruit different adaptor proteins leading to the activation of downstream signaling pathways (**Figure I.4.**) (Reviewed in [27,42]).

I.2.3. EGFR signaling

Phosphorylation of the EGFR C-terminus, by its autophosphorylation or by other kinases that have been activated, provides specific docking sites for SH2 or PTB domains of intracellular signal transducers or adaptors, leading to their colocalization and to the assembly of a multicomponent signaling module (reviewed in[22]). The C-terminal sequence divergence of each HER family member allows different proteins to preferentially associate with each specific EGFR heterodimer complex, therefore the multiplicity of signals that can emanate from EGFR varies considering the dimerization partner.

The main signaling pathways that are activated by EGFR are represented in **Figure I.4** and described below.

After stimulation, the major process that regulates the amplitude and the kinetics of EGFR signal transduction is endocytic removal of ligand-receptor complexes from the cell surface, and their subsequent sorting to degradation or recycling (reviewed in[43]). Despite the molecular events underlying this process are still not clear, it is believed that ligand-receptor complexes are internalized by clathrin mediated endocytosis. During this process EGFR is ubiquitinated by c-Cbl, a RING finger E3 ubiquitin ligase, which interacts directly or indirectly with the receptor through phosphoTyr1045 or through grb2[44,45]. This ubiquitination is necessary to sort the vesicles to lysosomal degradation.

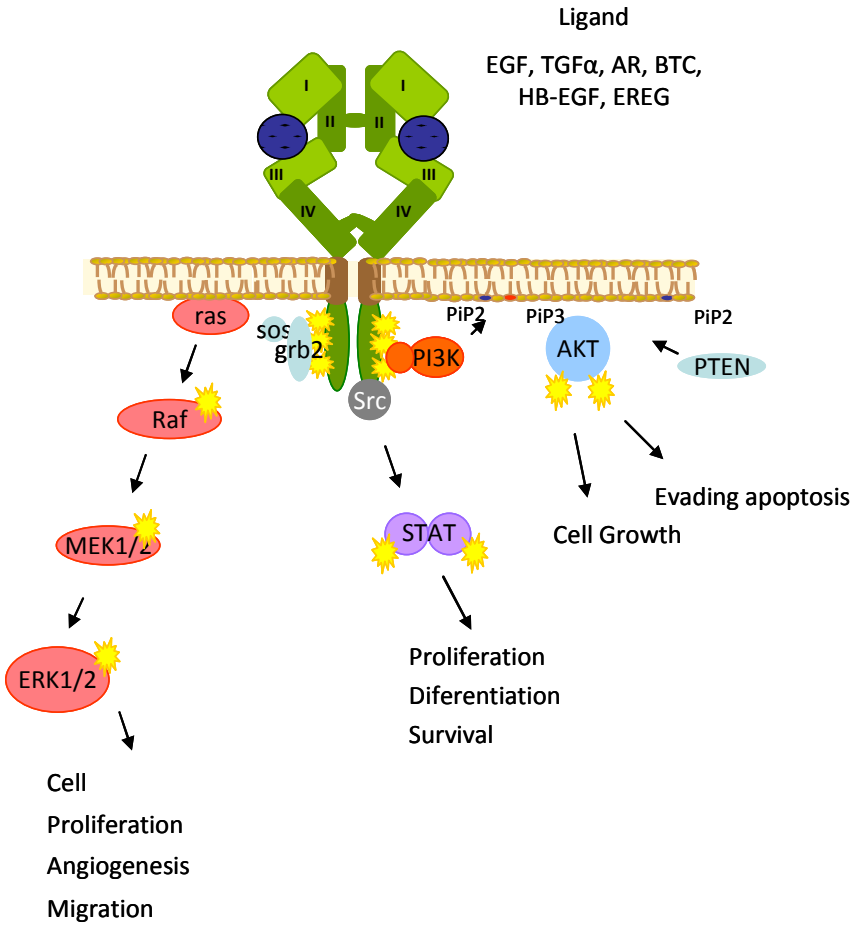


Figure I.4. EGFR signaling pathways. The scheme represent the classical signaling pathways that are activated upon EGFR stimulation; The RAS-RAF-MEK-ERK (MAPK) signaling cascade, PI3K- AKT pathway which is reversed by PTEN, the STATs signaling pathway and Src activation. The activation of all the signaling cascades promotes different cellular functions as proliferation, migration, angiogenesis, cell survival and cell growth.

I.2.3.1.RAS/MAPK pathway

One of the most important pathways activated by EGFR is the RAS-RAF-MEK1/2-ERK1/2 signaling pathway, also known as the ERK MAPK signaling pathway. The key player that leads to the activation of this signaling cascade is Grb2 adaptor protein[46]. In unstimulated conditions, Grb2 is constitutively bound to the GEF Sos and is localized in the cytosol. Following activation of EGFR, the SH2 domain of Grb2 can bind directly to the receptor by interaction with phosphoTyr1068 and phosphoTyr1086 or indirectly by interacting with Shc which is bound for its PTB domain to phosphoTyr1148 and phosphoTyr1173 of EGFR[47,48]. In either case, the relocalization of the Grb2/Sos complex to the receptor at the plasma membrane facilitates the interaction of GDP-RAF with Sos, resulting in exchange from GDP to GTP activated RAS.

There are three RAS genes (H-RAS, K-RAS and N-RAS) which encode for three G-proteins that are controlled by its GDP/GTP bound state. When RAS is activated and bound to GTP it binds with high affinity to one of the MAPKKK of the RAF family (RAF-1, A-RAF and B-RAF) which as a consequence it is activated[49]. Then, activated RAF phosphorylates the MAPKKs MEK1 and MEK2 that finally phosphorylate the MAPK ERK1/2, which is the effector kinase that translocates to the nucleus activating several transcription factors[50]. The RAS-RAF-MEK1/2-ERK1/2 pathway has generally been associated with increased proliferation, survival, angiogenesis, migration and invasion[51-53]. Activation of the MAPK signaling cascade also provides a negative feedback loop of the pathway by phosphorylating Sos and disrupting Grb2/Sos complex[54].

The role of the RAS/MAPK pathway in oncogenesis has been clearly demonstrated. In early 80's *RAS* protooncogene was identified by transforming mouse embryonic fibroblasts (MEFs) with the cDNA of different tumor cell lines. Southern blot analysis using viral transforming genes as probes for hybridization identified *HRAS* and *NRAS* genes as the responsible of the transforming process[55]. In addition, KRAS was found to induce cellular transformation of MEFs when mutated at position G12V[56,57]. This transforming mutation was present in cell lines and tumors from different origin, such as bladder cancer, colon cancer and lung cancer. Since then, *RAS* has been considered one of the most important oncogenes leading to tumor progression.

Due to the importance of the RAS-RAF-MEK-ERK pathway in triggering programs of cell proliferation and differentiation, and the relevance of *KRAS* mutations, *BRAF* gene was sequenced and screened for mutations in several cancer cell lines. Surprisingly, mutations in exon 15 (V600E and L596V) and exon 11 (G468A) were found in three different cell lines from melanoma and NSCLC. Subsequently, *BRAF* coding sequence was sequenced in 378 primary human cancers. V600E mutation was found in a 59% of melanomas, 5% of colorectal cancers, 11% of gliomas, and at low frequency in other cancer types. By *in vitro* experiments, it was observed that all these *BRAF* mutations induced high kinase activity of B-RAF protein and were also able to increase ERK1/2 phosphorylation. In addition, mutated B-RAF protein induced transforming capacity in MEFs. So, a clear role of *BRAF* mutations in promoting oncogenic processes in different tumor types was demonstrated[58].

Focusing in CRC, *KRAS* and *BRAF* mutations appear to be mutually exclusive. Approximately a 40% of CRC harbor *KRAS* activating mutations and a 20% of CRC tumors WT for *KRAS* harbor *BRAF* activating mutations. Interestingly, *KRAS* and *BRAF* mutations are detected in large adenomas and carcinomas, suggesting a role in the malignant progression rather than in tumor initiation[59,60].

I.2.3.2. The Pi3K/AKT pathway

The Pi3K/AKT signaling pathway plays a central role in diverse cellular functions such as survival, proliferation, growth, motility and metabolism [61].

Pi3Kinases are heterodimers formed by a regulatory subunit (p85) and a catalytic subunit (p110). When RTK as EGFR are activated by its specific ligand, Pi3K is recruited to the membrane by its regulatory subunit directly recognizing the activated form of the receptor by its SH2 domain and/or by adaptors proteins. The major binding partner of Pi3K is not EGFR, but HER3[62,63]. However, activation of Pi3K is observed in response to specific EGFR ligands through formation of EGFR/HER3 dimmers. Moreover, Pi3K can also be activated by RAS, which directly binds to the catalytic subunit[64]. Once in the membrane, the Pi3K catalytic subunit phosphorylates the membrane phospholipid PiP_2 into PiP_3. PTEN catalyzes the opposite reaction inhibiting Pi3K signaling. Once PiP_3 is formed, it recruits PDK1 and AKT in the membrane which is then phosphorylated by PDK1 at residue Thr308 and by mTOR complex 2 at residue Ser473 [65-67] .The phosphorylation of AKT is a key element to activate several signaling pathways which promote cell cycle progression, survival, cell growth

and metabolism. One of these cascades is the mTOR1 signaling pathway, which integrates nutrients and oxygen to promote protein synthesis and cell growth[68].

Several genetic alterations which increase the activation of the Pi3K/AKT pathway are encountered in human cancers. Mutations and overexpression of RTKs as well as mutations in *RAS* are common events in oncogenesis. Loss of PTEN function, usually by mutation or promoter methylation, has also been described[69,70]. *PI3KCA* gene encoding for the catalytic subunit p110α is frequently amplified or mutated; eighty per cent of *PI3KCA* mutations occur in exon 9 (E542K and E545K) and in exon 20 (H1047R). Both mutations give rise to a constitutive activation of AKT protein and have shown transforming capacity *in vitro* and *in vivo*[71].

I.2.3.3. The JAKs and STATs pathway

The STAT family is comprised for seven proteins including STAT1 to STAT4, STAT5a, STAT5b and STAT6. Of those, only STAT1, STAT3, STAT5a and STAT5b are known to play a role in cancer (reviewed in [72]).

STATs are transcription factors which dimerize and translocate to the nucleus upon specific receptor stimulation. Classically, STATs are activated by cytokine receptors through specific binding between STAT SH2 domain and the phosphotyrosines of the receptors. Activation is mediated by JAK kinases, which phosphorylate critical residues of STATs. Then, activated STATs form homo or heterodimers that translocate to the nucleus to activate gene transcription[73]. Different STAT target genes have been identified. In

particular, STAT3 and 5 regulate the expression of genes involved in cell cycle progression, survival, angiogenesis, migration and invasion, while STAT1 seems to function as a tumor suppressor by inducing cell cycle arrest and apoptosis[74-76].

The ligand dependent activation of STATs by EGFR is different of that observed by cytokine receptors. First, the ligand dependent phosphorylation of STATs does not require JAK kinases[77-79]. Second, STATs are constitutively associated with EGFR, however they require the tyrosine kinase activity of the receptor to become activated[78]. Moreover, Src family of kinases has also been implicated in STAT activation upon EGFR activation[80,81].

I.2.3.4. The Src family of kinases

Src family of kinases comprises 9 different members which are cytosolic tyrosine kinases that have been implicated in signal transduction from growth factor receptors such as EGFR (reviewed in[82]). However, it is not clear whether Src is a signal transducer downstream EGFR or a contributor of EGFR activation by promoting its phosphorylation. Both proteins share many substrates making difficult to discriminate between Src-mediated and EGFR-mediated signaling following stimulation of the receptor[83].

Src interacts with EGFR via a SH2 domain, although the exact residues on EGFR are unclear. It has also been observed that when Src is bound to EGFR it phosphorylates the receptor to provide docking sites for other signaling pathways as PI3K and STAT5b[81,84]. Thus, the roles of Src might be, first, to contribute to EGFR signaling by binding to the receptor and phosphorylating several cellular targets

that have been recruited to the receptor and second, phosphorylating the receptor itself to increase the docking sites for other proteins.

I.2.4. EGFR in cancer

In many different cancer types, the EGFR pathway is hyperactivated by a range of mechanisms, as overproduction of ligands, receptor overexpression and activating mutations of the receptor or downstream signaling pathways.

TGFα, one of the main EGFR ligands, is expressed by the stroma in androgen dependent prostate cancer and by tumor cells in advanced androgen independent prostate cancer. In pancreatic cancer, overexpression of this ligand correlates with tumor size and decreased patient survival. Moreover, in lung, ovary and colon, TGFα overexpression correlates with poor prognosis when coexpressed with EGFR. All these results suggest an important role in paracrine and autocrine signaling of this ligand activating EGFR dependent pathways[85,86].

Overexpression of EGFR itself has been observed in several tumor types as HNSCC, breast cancer, bladder cancer, prostate cancer, NSCLC and gliomas, being associated with worse prognosis of the patients[87,88]. In addition, EGFR overexpression in mice mammary gland induces tumor formation and inhibits differentiation[89].

Different genetic alterations of the receptor have also been described to occur in cancer. In particular, a deletion in the extracellular domain of the receptor that leads to a aberrant transcript variant, EGFRvIII, is observed in 40% of gliomas[90]. This variant gives rise to a

constitutively active protein which has transforming capacity despite the presence of the ligands[91,92]. Moreover, activating mutations and deletions in the TK domain of the receptor have been found in NSCLC[93-95] and predict sensitivity to TK inhibitors. All these data suggest that EGFR signaling is one of the main pathways promoting tumor growth in different solid tumors.

In colorectal cancer, EGFR overexpression has been associated with poor differentiation, reduced survival and increased risk of metastasis[96,97]. EGFR expression is found in approximately 70% of CRC and its gene amplification occurs in a 30 to 50% of colorectal tumors[98,99], however, activating mutations of the receptor are rare events in this tumor type[100].

I.2.5. Drugs targeting EGFR

The concept of EGFR as a therapeutic target was developed from several key observations made from the laboratory and from the clinics. Preclinical studies showed that EGFR activation promoted multiple tumorogenic processes and it was found that monoclonal antibodies against EGFR could inhibit EGF induced cell proliferation[101]. And, as clinical evaluation showed that EGFR expression or its ligands was elevated in solid tumors and was associated with aggressive disease and poor outcome[86], EGFR was considered a promising target for cancer treatment.

Figure I.5. EGFR inhibitors. Scheme of the approaches to inhibit EGFR approved for cancer treatment; EGFR monoclonal antibodies, cetuximab and panitumumab, and TK inhibitors, gefitinib, erlotinib and lapatinib.

Nowadays, two therapeutic approaches exist to inhibit EGFR signaling; small molecules inhibiting the TK domain of the receptor and monoclonal antibodies which prevent ligand binding and receptor stimulation. **(Figure I.5.)**

I.2.5.1. TK inhibitors

TK inhibitors are small molecules of low molecular weight that target and inhibit specifically the ATP binding site of the TKR[102,103]. Three

different EGFR TK inhibitors have been approved for the treatment of different cancer types:

- Gefitinib: Is a specific EGFR TK inhibitor approved as first line treatment of NSCLC patients with tumors harboring activating mutations of the TK domain of the receptor[104].
- Erlotinib: Is a specific EGFR TK inhibitor approved as maintenance treatment in patients with NSCLC who do not progress after four cycles of chemotherapy[105] and as second line treatment of NSCLC patients[106]. It is also approved for treatment of advanced pancreatic cancer in combination with gemcitabine[107].
- Lapatinib: Is a dual EGFR/ErbB2 TK inhibitor. It is approved for treatment as second line treatment in combination with capecitabine of HER2 overexpressing breast cancer patients after failure to trastuzumab therapy[108] or as first line treatment in combination with letrozole in Estrogen Receptor positive and HER2 positive breast cancer[109].

I.2.5.2. Monoclonal antibodies

There are different monoclonal antibodies approved for the treatment of different diseases. The effect of therapeutic antibodies is mediated through antagonizing the receptor dependent signaling transduction. Moreover, its effects might be also mediated by the antibody-dependent cell mediated cytotoxicity (ADCC)[110], an immune response in which antibodies, by coating target cells, make them vulnerable to be attacked by immune cells as NK cells or phagocytes.

Monoclonal antibodies against EGFR compete with EGFR ligands for the binding of the receptor, therefore preventing its stimulation.

There are currently two different anti-EGFR moAb approved in Europe for clinical use:

- Cetuximab: Approved as first line treatment for mCRC in combination with 5-Fluorouracil and Irinotecan (FOLFIRI) or Oxaliplatin (FOLFOX) in *KRAS* WT tumors[111-113]. It is also approved as first line treatment of advanced HNSCC in combination with radiotherapy or chemotherapy[114].
- Panitumumab: Approved as first line treatment for mCRC in combination with FOLFOX and as second line treatment in combination with FOLFIRI in *KRAS* WT tumors[115,116].

I.3. ANTI-EGFR MONOCLONAL ANTIBODIES FOR THE TREATMENT OF mCRC

I.3.1. Cetuximab as a therapeutic agent in mCRC

I.3.1.1. Cetuximab in preclinical models

In 1983, four mouse hybridomas secreting monoclonal IgG antibodies to EGFR of A431 cells were obtained independently from four fusion experiments[101]. One of them, the moAb 225, was the murine precursor of chimeric C225, later on termed cetuximab. It was observed that the antibody competed with EGF for EGFR binding sites and could antagonize the stimulation of the TK activity of the receptor[101,117]. (**Figure I.6**).

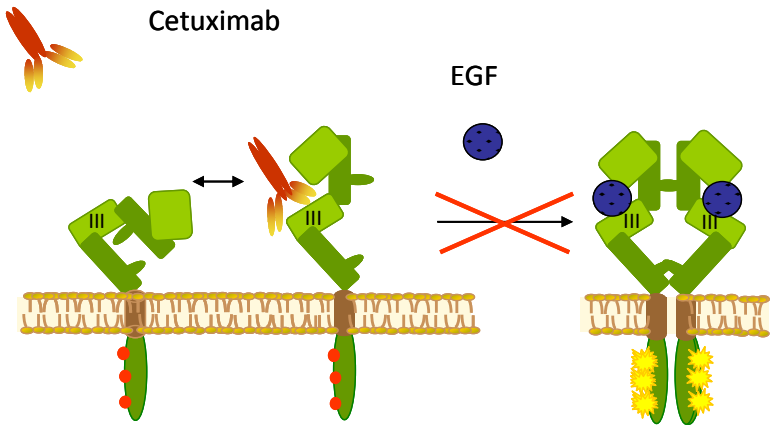


Figure I.6. Cetuximab prevents EGFR from adopting the extended conformation. Model for inhibition of ligand-induced dimerization by cetuximab. In addition to blocking the domain III ligand binding site, cetuximab prevents the receptor from adopting the extended conformation required for high-affinity ligand binding and dimerization.

This monoclonal antibody from mouse origin was used in a phase I study to treat patients with squamous cell carcinoma (SCC), which invariably expresses high levels of EGFR[118]. The study concluded that the treatment with the anti-EGFR moAb was safe at the doses tested; however all the patients produced anti-murine antibodies. To avoid this problem, 225 was chimerized to the human IgG1 constant region, resulting in C225 or cetuximab, and their biological effects on A431 tumor xenografts in nude mice were compared[119]. It was expected that the biological characteristics of both antibodies would be similar. However, they found that cetuximab was more effective inhibiting tumor growth than the murine counterpart, probably due to higher affinity to bind EGFR. Several studies were done to elucidate the cellular mechanisms by which cetuximab was doing its antitumor activity. It was observed that both cetuximab and 225 were able to cause cell cycle arrest in G1 phase by increasing p27KIP1 protein in cell lines from different origin, such as CRC, prostate cancer and HNSCC[120-122].

There was also data attributing anti-angiogenic properties to cetuximab, as it was able to inhibit the secretion of angiogenic factors induced by EGF, such as VEGF and IL-8, resulting in a decrease of blood vessels and metastasis in tumor xenografts[123-125]. The role on metastasis was also explained by the ability of cetuximab to decreases MMP production with a significant reduction of *in vitro* tumor cell invasion and a decrease in tumor growth and metastasis in nude mice[126,127]. This inhibition of angiogenesis, invasion and metastasis may explain why cetuximab is often more effective in *in vivo* than in *in vitro* models. Despite that in most of cell lines tested cetuximab induced

cytostatic effects, it appeared to induce apoptosis in some cell lines from CRC and SCC[122,128].

Interestingly, apart of the effects observed as a single agent, C225 was also able to enhance the effects of chemotherapy and radiotherapy in combined treatments. In xenografts of tumor cells expressing high levels of EGFR, A431 from SCC and MDA-MB-468 from breast cancer, moAb 225 was able to enhance the effects of doxorubicin[129]. In addition, same effects were observed in combination with topotecan when treating xenografts from CRC cell lines. While mice treated with topotecan and C225 as single agents died within 6 and 10 weeks respectively, all mice treated with a combination of both agents were still alive at 14 weeks after cell injection, 20% of them lived more than 19 weeks[130]. Other studies demonstrate the ability of cetuximab to potentiate the effects of radiotherapy in A431 xenografts[131,132].

I.3.1.2. Cetuximab in the clinical practice

The efficacy of cetuximab in preclinical models encouraged the initiation of many clinical trials. In 2000, the first study in patients using cetuximab was published. This study included three consecutive phase I trials where C225 was given as single dose, weekly multiple dose and in combination with cisplatin (only for HNSCC and NSCLC patients)[133]. Patients with different epithelial tumors were included in the trial and as in preclinical models C225 had only shown activity in cell lines expressing EGFR, all of them harbored EGFR expressing tumors as determined by IHC. Despite this first trial was designed to establish the immunogenicity of C225 and to determine the recommended doses, it was also observed that some patients

experienced disease stabilization and two patients had a partial response.

After this first study with C225, several phase II trials were addressed to different kind of tumors.

The study of cetuximab as treatment for mCRC patients started in 2004. In that moment, only chemotherapeutic agents, such as 5-Fu, capecitabine, oxaliplatin and irinotecan, were used for the treatment of mCRC. However, once a patient became refractory to the treatment, no more options were available. So there was an urgent need to find new agents for this disease. As EGFR expression was observed in a 25-75% of colorectal tumors[134], C225 was a candidate to be used for the treatment refractory mCRC patients as a single agent or in combination with chemotherapy.

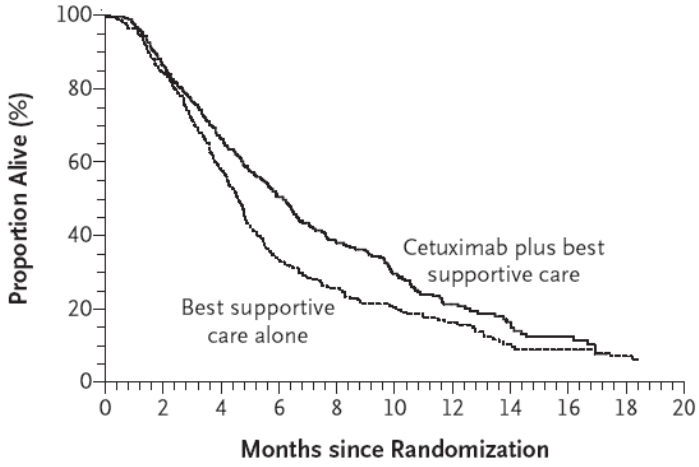
Two phase II trials were performed evaluating the efficacy of cetuximab in refractory mCRC[17,135]. Both included patients with mCRC tumors refractory to irinotecan-based therapy and harboring EGFR expression.

First, Saltz et al. analyzed cetuximab activity as single agent in 57 patients, observing 5 partial responses and 22 stable diseases. The median time to progression was 1.4 months, being 4.2 months in those patients who had partial responses to cetuximab, and the median overall survival was 6.4 month since the beginning of cetuximab treatment.

In the other hand, Cunningam et al. analyzed the efficacy of cetuximab as monotherapy or combined with irinotecan in 329 patients with refractory mCRC. Overall survival was 6.8 months when cetuximab was used as single agent and 8.1 months in combination with chemotherapy. Response rates and time to progression were

higher in the combination group, therefore they concluded that a novel option for refractory mCRC should be irinotecan plus cetuximab, considering cetuximab as single agent for those patients who have irinotecan derived side effects. Interestingly, in both studies it was observed that there was no apparent relationship between cetuximab efficacy and the level of EGFR expression determined by IHC, so they wondered if tumors without detected EGFR expression would be also responsive to the treatment, and that it should be necessary to find molecular determinants of cetuximab response to determine which patients would achieve benefit from the treatment.

Considering these observations, in 2005 a study addressing whether cetuximab could be used in tumors that do not have detected EGFR expression by IHC[136] was published. Pharmacy computer records were reviewed to identify all patients who received cetuximab in Memorial Sloan-Kettering Center in a non-study setting. Pathology slides were reviewed to find those patients that were negative for EGFR expression by IHQ, and 16 patients were identified. Of these EGFR negative patients, 14 received cetuximab plus irinotecan therapy and 2 cetuximab monotherapy. Interestingly 4 of the patients achieve major objective responses, suggesting that a 25% of tumors negative for EGFR expression would have benefit from cetuximab based regimens, which is exactly the same percentage as for tumors that express EGFR, therefore EGFR expression by IHC was not anymore a determinant to exclude or include patients to receive cetuximab treatment.



No. at Risk											
Cetuximab plus best supportive care	287	245	189	136	87	60	37	20	13	4	1
Best supportive care alone	285	235	157	85	58	37	26	15	11	8	4

Figure I.7. mCRC patients benefit from cetuximab treatment. Kaplan–Meier Curves for overall survival of patients treated with best supportive care and cetuximab plus best supportive care[137].

In 2007, it was published that cetuximab improved overall survival, Progression Free Survival and preserved quality-of-life measures in patients with colorectal cancer in whom other treatments had failed, as compared with best supportive care alone[137]. As a result, cetuximab monotherapy was approved for chemotherapy refractory mCRC. (**Figure I.7**).

In 2009, a multicenter phase III study to determine the efficacy of cetuximab as first line treatment in combination with FOLFIRI in mCRC was published[111]. In this study, 599 patients received cetuximab in combination with FOLFIRI as first line treatment and 599 FOLFIRI alone. The addition of cetuximab to FOLFIRI reduced

the risk of disease progression by 15% and increased the response rate by nearly 10%. However, there was no significant difference in overall survival between the groups. Similar results were achieved in a phase III trial combining FOLFOX with cetuximab[113]. While the addition of cetuximab did not increased overall survival, it increased overall response rates. Interestingly, adding cetuximab to FOLFIRI increased the rate of resection of metastasis, but whether it improved the potential for cure or long-term survival was unknown. Since then, cetuximab plus chemotherapy was approved as first-line treatment for mCRC.

I.3.2. Panitumumab as a therapeutic agent in mCRC

I.3.2.1. Panitumumab in preclinical models

Until early 1990's all the antibodies candidates for a clinical use were mouse moAbs that when administered in humans elicited the production of human anti-mouse antibodies, resulting in a rapid clearance, limited efficacy, immunogenicity and allergic reactions[138], which were especially problematic in the treatment of chronic and recurrent human diseases. These issues forced the development of several approaches to generate more human-like antibodies.

Panitumumab, formerly known as E7.6.3 and ABX-EGF, is a fully human IgG2 antibody produced in a xenomouse strain, which produces human instead of mouse immunoglobulins. These strains are generated in mouse embryonic stem cells, in which its antibody production machinery has been inactivated by gene-targeted deletion and the human immunoglobulin loci has been introduced by stable

cloning[139]. Panitumumab was raised, as cetuximab, by immunizing a xenomouse using A431 cells. Fusion of B cells from the immunized xenomouse strain with mouse myeloma cells yielded to 30 hybridomas which produced specific anti-EGFR moAb's. Panitumumab was chosen based on its high binding affinity, specificity and its ability to block ligand binding and receptor phosphorylation[140].

Several studies showed that panitumumab had similar effects to cetuximab at a preclinical level. Panitumumab monotherapy was able to prevent and inhibit tumor growth of xenograft models of EGFR overexpressing cells, as A431 and MDAMB468, and low EGFR expressing cells, as BxPC3 and HT-29[140,141]. Interestingly, not all EGFR-expressing tumors were sensitive to panitumumab, suggesting that EGFR expression alone was not sufficient to predict its response. Moreover, in combination with various standard chemotherapeutic agents panitumumab alo demonstrated and additive effect *in vivo*[140]. The specific binding site to EGFR was though to be similar to cetuximab, however this remained unpublished.

I.3.2.2. Panitumumab in the clinical practice

The first clinical trial with panitumumab was published in 2004. The aim of this study was to prove the safety and pharmacokinetics of panitumumab, and the trial enrolled 88 patients with metastatic renal cell carcinoma[142]. In this study it was concluded that the antibody was well tolerated at all conditions analyzed, albeit an increase of skin rash was observed at higher doses.

Due to the results observed with cetuximab for mCRC, soon, a phase III trial of panitumumab plus best supportive care was done in

patients with chemorefractory mCRC[18] . The trial enrolled 463 patients and, as observed with cetuximab, panitumumab significantly improved PFS with manageable toxicity.

As with cetuximab, the first studies performed with panitumumab considered EGFR positivity by IHC analyzed in the trials, but again, it was demonstrated that there was a lack of correlation between EGFR status and response to anti-EGFR antibodies[143].

From June 2006 to March 2008, a randomized phase III trial was conducted in 1186 mCRC patients comparing as second-line treatment of panitumumab with FOLOFIRI or FOLFIRI alone[116]. The results regarding overall survival were not significant; however, the addition of panitumumab increased the response rate from 10% in the FOLFIRI group, to a 35% in the combination group. More recently, benefit was observed when combining panitumumab with FOLFOX as first-line treatment[115], as panitumumab improved Progression Free Survival from 8 to 9.6 month. Although no differences were observed considering overall survival, panitumumab has been approved as first-line treatment for mCRC.

I.4. CHALLENGES IN ANTI EGFR moAb THERAPY

Despite the advances in mCRC treatment, prognosis of patients remains poor with a median overall survival from 18 to 21 months. Since the first studies done with anti-EGFR antibodies until now, only 10 to 20% of all mCRC patients benefit from these therapies, suggesting that 80% of tumors have intrinsic or primary resistance to these agents [137]. (**Figure I.8**)

Figure I.8. Primary resistance to cetuximab. From all mCRC patients that are treated with cetuximab, 90% of tumors have primary resistance to the treatment (light red) and only 10% (green) respond to the treatment. (Figure adapted from[144])

Moreover, those tumors that initially respond to the treatment eventually become resistant by the development of secondary or acquired resistance, in the best of the cases within 12-18 months[137]. Therefore, to identify the patients who are candidates to receive anti-EGFR treatment and to understand the mechanisms by which tumors become resistant are the main challenges to select those mCRC patients that will benefit and to develop strategies to overcome resistance (reviewed in[144]).

I.4.1. Predictive factors of response to anti-EGFR mAbs

To study the mechanisms and find molecular markers involved in sensitivity to anti EGFR mAbs, would make possible to identify those patients with mCRC who really subject to benefit from the treatment. (**Figure I.8**)

I.4.1.1. EGFR expression

When the first clinical trials with cetuximab were performed, it was considered that EGFR expression within the tumor was an essential requirement to benefit from the treatment, as discussed in a previous section. EGFR expression determined by IHC was the molecular marker which determined whether a patient was a candidate or not to receive cetuximab[17,133,135]. Although the IHC staining protocol was standardized, no correlation between the levels of EGFR expression and tumor response to cetuximab was observed. Approximately 20% of tumors responded. It was also observed that 20% of tumors determined EGFR negative also responded to the treatment[136], so EGFR expression by IHC was not considered anymore a marker of cetuximab response.

The lack of correlation between EGFR expression and cetuximab response could be attributed to:

- Technical problems related to IHC determination.

  Although a standardized protocol of IHC has been proposed (EGFR pharmDX^{tm} Kit, DAKO, Denmark), all issues concerning tissue processing may influence the pathologist determination of EGFR expression within different hospitals and may limit the

efficacy of EGFR determination by IHC as a marker of cetuximab response. The fixative used when processing tumor sample can influence the result, as variation in the determined level of EGFR expression was demonstrated when comparing samples fixed with different fixatives[145]. Moreover, the immunoreactivity to anti-EGFR is inversely correlated with the storage time of unstained slides.

- Lack of correlation of EGFR expression in the primary tumor and the related metastasis.

  EGFR expression is usually determined in the biopsy of the primary tumor performed when the patient is diagnosed; however cetuximab is given to treat metastatic disease. It has been observed that some primary mCRC tumors which are found positive for EGFR are negative in the corresponding metastatic site[146]. These observations have been found in mCRC but also on other tumor types[147], suggesting that within the metastatic process there is a gain of information not given by the primary tumor which may limit the clinical decision on treatment.

Due to the lack of a predictive role of EGFR determination by IHC as a marker of cetuximab response other markers have been proposed to identify those patients subject to benefit from the treatment.

I.4.1.2. EGFR gene amplification

While 10% of NSCLC lung cancers present activating mutations within EGFR TK domain which predict sensitivity to gefitinib[94], these mutations occur at a very low frequency in colorectal cancers[100].

However, an important percentage of colorectal tumors display an increase in *EGFR* gene copy number, suggesting tumor addiction to EGFR signaling mediated by this mechanism.

The first study trying to find a correlation between *EGFR* copy number and cetuximab response was published in 2005[99]. In a cohort of 31 mCRC patients treated with cetuximab, it was observed that 25% of tumors had an increase of *EGFR* copy number. Interestingly, none of the tumors that did not respond to cetuximab had an increase in the copy number, while 8 of the 9 responders had. This observation suggested that those tumors without an increase in EGFR gene copy number were more likely to be resistant to the treatment.

These results were further confirmed in a larger cohort and also using panitumumab[98,148], where it was observed that tumors with *EGFR* gene amplification were more likely to respond to panitumumab treatment.

Despite the reported correlations with *EGFR* copy number and likely to respond to EGFR moABs, several discrepancies have been observed; the increased EGFR gene dosage does not seem to translate into an increase in the protein levels[98], and the results obtained when analysing *EGFR* copy number by quantitative PCR have been inconclusive[99,149]. Moreover, FISH scoring system shows high interlaboratory variability complicating its clinical evaluation. Therefore, how and why the copy number of *EGFR* is correlated with better response to anti EGFR moAbs remains unclear and needs further validation to be used as a predictive marker to select patients.

I.4.1.3. mRNA expression of EGFR ligands

Due to the lack of established markers of cetuximab sensitivity related to the own EGFR, an exploratory clinical trial was done to systematically identify biomarkers associated with disease control to cetuximab treatment[149]. In this trial, the metastatic lesion, usually from the liver, was biopsied and blood samples from the patients were obtained from 103 patients with mCRC before starting cetuximab monotherapy. By RNA expression arrays several genes were differentially expressed between those tumors that were sensitive and resistant to cetuximab. Interestingly, amphiregulin and epiregulin mRNA, which are two EGFR ligands, were more expressed in those tumors sensitive to cetuximab than in those resistant. No correlation was found when analyzing the ligands by ELISA in the blood samples. Although the results in mRNA levels were significant, they lacked clinical utility, because the RNA extracted was from frozen tissue from metastatic lesions, which is a difficult material to obtain in the hospitals daily routine. A similar study was done determining amphiregulin and epiregulin levels in FFPE samples from the primary tumor, which has more clinical applicability[150]. The results were reproduced; EGFR ligands expression in primary tumors significantly predicted outcome in mCRC treated with cetuximab. These results suggested that those tumors with more amphiregulin and epiregulin expression would be more addicted to EGFR signaling and cetuximab would be mediating its effects by preventing ligand binding to the receptor.

However, the levels of expression of amphiregulin or epiregulin are difficult to asses systematically, so at present, these markers cannot be used to select patients eligible for cetuximab or panitumumab therapy.

I.4.2. Molecular markers of resistance to anti EGFR mAbs

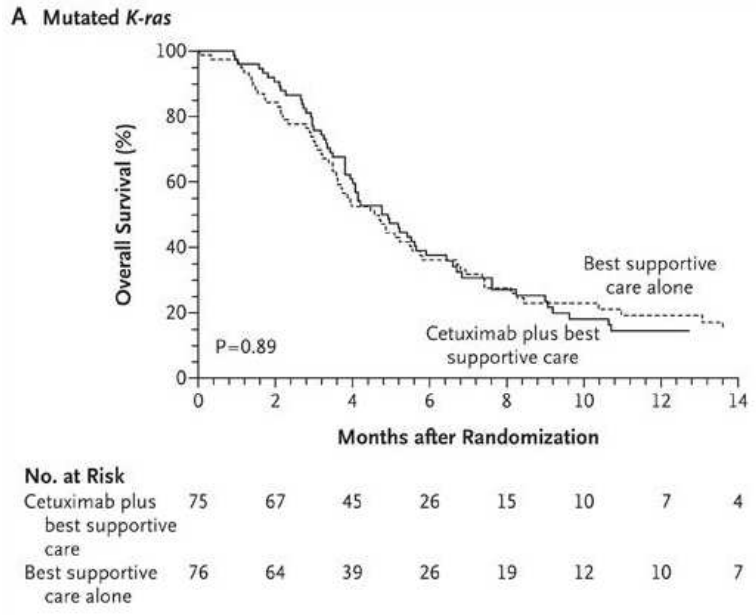
To study the mechanisms and find molecular markers involved in resistance to anti EGFR mAbs, would make possible to identify those patients who will not benefit from the treatment (**Figure I.8**).
In addition to molecular alterations of the *EGFR* gene itself or EGFR ligands, oncogenic activation of EGFR downstream effectors has also been investigated considering clinical response to cetuximab and panitumumab.

I.4.2.1. Mutational status of KRAS

KRAS-MAPK signaling cascade and the PI3K-AKT axis have been described to be altered in human tumors, therefore, any alteration giving rise to a constitutive activation of these pathways would overcome anti-receptor effects leading to tumor resistance.
Initial retrospective analysis on small number of patients treated with cetuximab led to the suggestion that patients with activating mutation of *KRAS* do not benefit from cetuximab treatment[151]. The activating mutation was present in around 40% of mCRC tumors, and most patients with the mutation progress to cetuximab treatment. Moreover, the introduction of mutated *KRAS* gene in the DiFi cell line, which is highly sensitive to cetuximab, induced resistance to the treatment[152].

On October 2008, in a retrospective analysis of 394 mCRC patients who had been randomly assigned in a clinical trial to receive cetuximab plus best supportive care or best supportive care alone, it was assessed whether *KRAS* mutation status was associated with survival in the cetuximab and the supportive care groups (**Figure I.9**). Interestingly it was found that 42% of the tumors presented *KRAS* mutations, and that the mutational status was associated with cetuximab benefit. Cetuximab only improved overall survival and time to progression in patients with *KRAS* WT tumors. Those patients with mutated tumors did not benefit from cetuximab[112].

Similar results were obtained with panitumumab when analysing its efficacy in *KRAS* mutated tumors[153]. The relevance of *KRAS* status was not only significant when anti-EGFR antibodies were given as single agents, also when cetuximab was given in combination with chemotherapy[111,113,154].



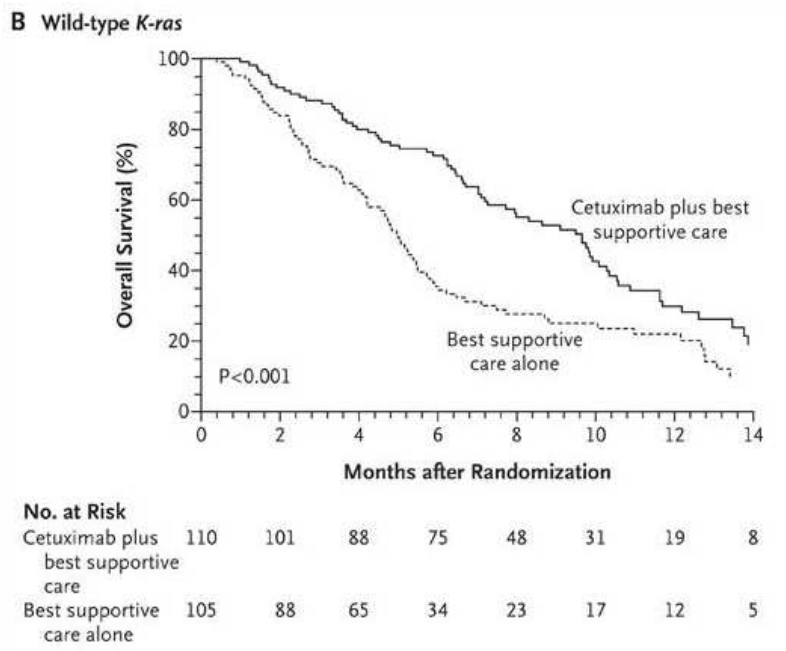


Figure I.9. KRAS mutated as a mechanism of primary resistance to cetuximab. Panel A shows Kaplan–Meier Curves for Overall Survival according to results from patients with mutated *KRAS* tumors, and panel B for patients with wild-type *KRAS* tumors. Cetuximab as compared with best supportive care alone was associated with improved overall survival among patients with WT *KRAS* tumors but not among those with mutated *KRAS* tumors[112].

Considering all this clinical data, in January 2009, the American Society of Clinical Oncology recommended that if *KRAS* codons 12 or 13 are mutated, patients with mCRC should not receive anti-EGFR antibody based therapy as part of their treatment[155].

I.4.2.2. Mutational status of BRAF

KRAS mutations occur in 40% of mCRC tumors, while only 10% of mCRC respond to anti-EGFR antibodies. Therefore, the identification

of other markers of resistance to anti-EGFR therapies remained important to identify those patients that should not receive the therapy. Recent studies have focused on other EGFR signaling pathway alterations, as *BRAF* mutations and oncogenic deregulation of the PIK3CA/PTEN signaling.

Interestingly, *KRAS* and *BRAF* mutations are known to be mutually exclusive in colorectal cancer[59], so the presence of *BRAF* mutations could lead to resistance to anti-EGFR antibodies in *KRAS* WT tumors. According to that, in a retrospective study that was done in 113 patients who received cetuximab or panitumumab, *BRAF* mutations were detected in 11 of 79 *KRAS* WT tumors, and none of them responded to anti-EGFR antibodies[156]. Moreover the introduction of the activating BRAF mutant V600E in cell lines impaired the therapeutic effect of cetuximab or panitumumab, and treatment with sorafenib, a BRAF inhibitor, restored cetuximab sensitivity in *BRAF* mutant cell lines[156]. Furthermore, in another retrospective study of 173 mCRC patients *BRAF* mutations were found in 5 patients, and again they accounted for lack of cetuximab response and of interest, they were associated with shorter overall survival[157]. However, even these results on BRAF are promising, larger prospective analysis are needed to confirm these data.

I.4.2.3. Oncogenic activation of Pi3K/AKT pathway

In addition to KRAS and BRAF, EGFR also activates the Pi3K signaling pathway, which can also be deregulated by the presence of activating mutations of Pi3K catalytic subunit or by PTEN loss of function. Therefore, the role of this pathway on the response to

cetuximab or panitumumab is currently being investigated. However, the published results are somehow contradictory.

Approximately 10% of mCRC tumors harbour *PIK3CA* oncogenic mutations and PTEN expression is lost in around 20%. Several studies have been conducted to analyze its role in predicting cetuximab or panitumumab resistance. The results are not yet conclusive, but the tendency is that oncogenic activation of this pathway might lead to resistance[157-160]. However, several issues have to be solved before considering them as markers to use in the daily clinical practice. First of all, Pi3K and PTEN alterations can coexist with *KRAS* or *BRAF* mutations, making more difficult to establish its independent role in cetuximab resistance[161]. Moreover, PTEN function is evaluated by IHC, and the lack of standardization in the clinical application of its analysis is likely to give equivocal results. Thus, considering these issues these markers are not yet ready to be used in the clinical practice.

I.4.3. Acquired resistance to anti-EGFR mAbs

All tumors that initially respond to anti-EGFR mAbs finally become refractory, even the best responses are transient and they do not last longer than 12 to 18 months[17,137](**Figure I.10**).

Nothing is known about why, after an initial reduction of volume, the mCRC tumors eventually begin to regrow and concomitantly become refractory to further anti-EGFR treatment. The timeframe of these events may be explained by Darwinian selection with the emergence of resistant clones. However, the difficulty to obtain tumors samples after cetuximab or panitumumab failure has impaired the research to

understand this process and nothing is known at a clinical level of why it is occurring.

One possible explanation would be the selection of cells harboring mutations which are already known to account with cetuximab or panitumumab resistance, as *KRAS*, *BRAF* or *PIK3CA* mutations, however, this information is still lacking.

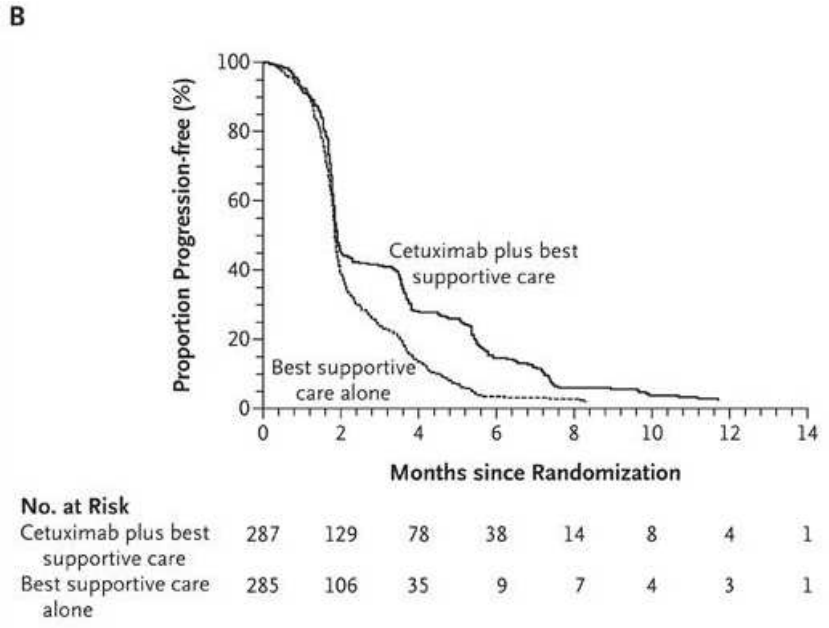


Figure I.10. mCRC patients that initially respond to cetuximab, finally become refractory. Kaplan–Meier Curves for progression-free survival of patients treated with best supportive care and cetuximab plus best supportive care[137].

Another explanation that has been suggested is that those tumors which initially depend on EGFR for their growth and survival alternatively overexpress or overactivate another RTK to maintain the same signaling pathways activated despite EGFR inhibition, therefore becoming resistant. This model has been observed in NSCLC as a

mechanism of gefitinib resistance, in where c-met amplification and or transactivation of other HER family members account for the resistance[162-164]. These results suggest that combining different RTK inhibitors could overcome the acquisition of anti-EGFR acquired resistance.

Due to the lack of clinical evidence of most of studies done to understand the secondary resistance, it is clear that clinical studies designed to ethically obtain biopsies, mainly from liver metastasis, from patients who have become refractory to therapy will be pivotal to address this question.

HYPOTHESIS

Acquired resistance to cetuximab treatment in mCRC patients is caused by genetic and molecular alterations that emerge or occur due to the selective pressure imposed to malignant cells by drug exposure. The identification of the mechanisms that lead to the acquisition of the resistance is an essential first step to develop clinical strategies to prevent or revert resistance.

OBJECTIVES

The general objective of this PhD thesis was to study and identify mechanisms that lead to cetuximab acquired resistance in mCRC.

The specific objectives were:

1. **To study the mechanisms of acquired resistance to cetuximab in CRC cell lines.**
   - To generate cetuximab-resistant CRC cell lines derived from a parental highly sensitive CRC cell line continuously exposed to cetuximab.
   - To study the molecular factors responsible of resistance by comparing different genes and/or protein expression in the parental versus the resistant cell lines.
   - To confirm the molecular mechanism responsible of resistance by functional studies.

2. **To identify pharmacological approaches to overcome the resistance**

3. **To characterise the molecular mechanism of cetuximab acquired resistance in tumor samples from CRC patients.**
   - To collect tumor samples from mCRC patients before cetuximab treatment and at the moment of treatment failure.
   - To validate the results obteined in cell lines in the tumor samples.

RESULTS

R.1. GENERATION OF CELLS WITH ACQUIRED RESISTANCE TO CETUXIMAB

R.1.1. Cell culture modeling of acquired resistance to cetuximab in colorectal cancer

All tumors that initially respond to cetuximab finally become resistant[137]. We believe that chronic exposure to cetuximab leads to clonal selection of those tumor cells that can overcome the therapy leading to relapse. To identify mechanisms of acquired resistance to cetuximab in colorectal cancer, we used a widely validated translational model to study the acquisition of resistance to targeted therapies in cancer[162,165,166]. Briefly, the model consists in three different parts. In the first part we expose a cetuximab sensitive colorectal cancer cell line to the drug chronically until few clones acquire resistance and grow despite the presence of cetuximab in the media. In the second part, once we have obtained stable cetuximab resistant clones, we compare the original sensitive cell line with the resistant clones to identify the molecular mechanism responsible of the resistance. And finally, the third part consist in confirming the results in the clinical setting by analysing the molecular alteration in patients that initially respond to cetuximab but finally become refractory, comparing the biopsy obtained at the moment of the diagnosis with a biopsy obtained at the moment of relapse. A scheme of the proposed model can be observed in **Figure R.1.**

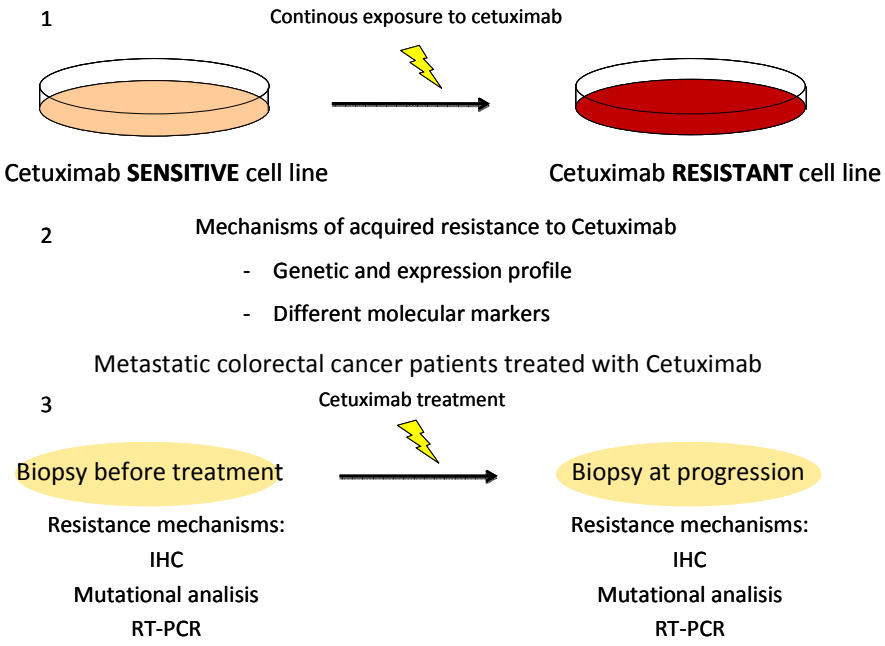


Figure R.1. Schematic representation of the working model

In order to choose the colorectal cancer cell line to work on with the model, we have treated different colorectal cancer cell lines at growing concentrations of cetuximab during 48h and we have checked the effects of the drug by MTS assay. As observed in **Figure R.2a**, the only CRC cell line that was sensitive to the therapeutic antibody was DiFi cell line. We have checked by WB the ability of cetuximab to inhibit EGFR signaling pathways in DiFi cells, and as observed in **Figure R.2b**, cetuximab inhibits ERK1/2 and AKT phosphorylation in a dose dependent manner which correlates with the results observed by MTT assay. Cetuximab treatment at 1μg ml^{-1} is the minimal dose where we observe the maximum effect on cell

proliferation, and consequently, ERK1/2 and AKT are completely inhibited at this dose. These molecular effects were maintained at least for 24 hours, **Figure R.2c**.

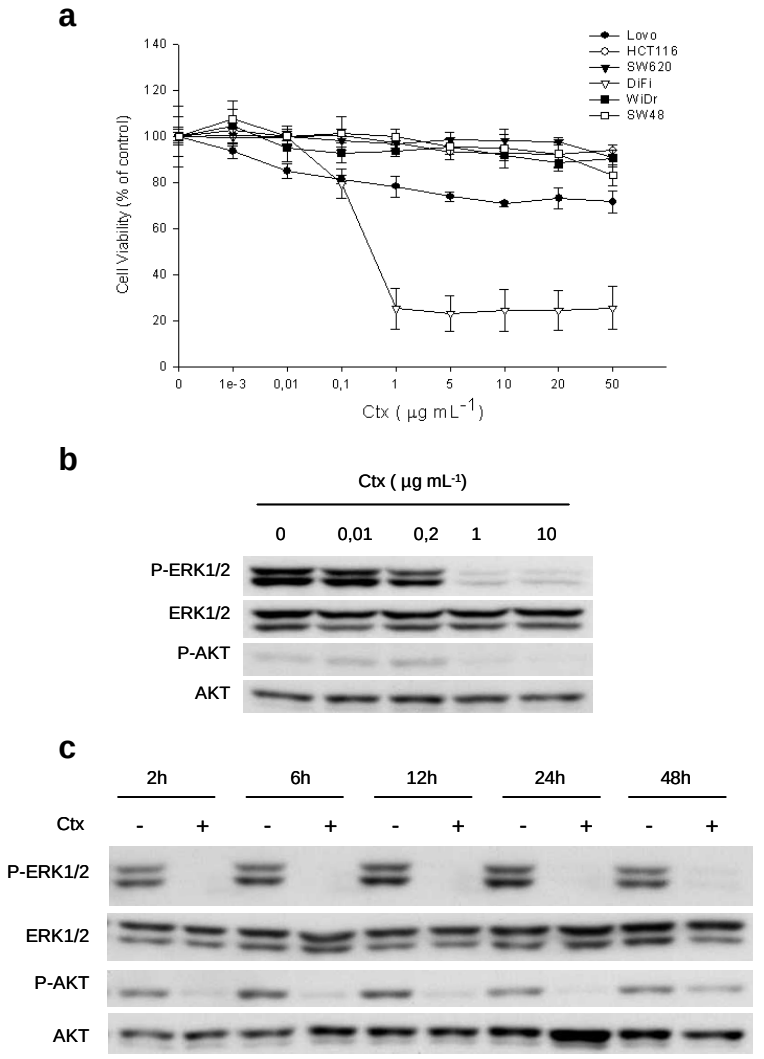
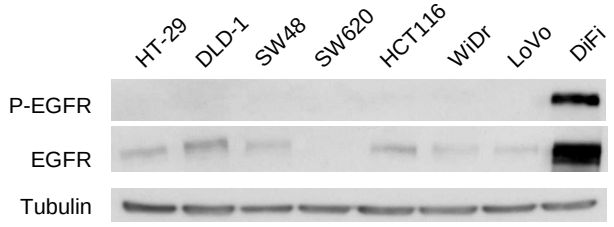


Figure R.2. DiFi cell line is sensitive to cetuximab (ctx) treatment which effectively blocks EGFR dependent pathways. a, MTS assay done in different CRC cells lines treated with the indicated concentrations of ctx during 48h. Error bars represent the standard deviation of three independent experiments. **b,** WB analysis of P-ERK1/2, ERK1/2, P-AKT and AKT in Difi cells treated for two hours at the indicated concentrations of cetuximab.**c,** WB analysis of P-ERK1/2, ERK1/2, P-AKT and AKT in a time-course of DiFi cells treated with ctx 10µgmL^{-1}.

As cetuximab is designed specifically to bind with high affinity to EGFR, we have checked the amounts of EGFR protein and its basal status of phosphorylation by WB in the panel of CRC cell lines. **Figure R.3a** shows how despite most of colorectal cancer cell lines express EGFR, DiFi cell line overexpress the protein and is the only cell line with high basal activity of the receptor analyzed by the phosphorylation status of the residue Tyr1068, one of the main effector residues of EGFR. Also, as described in the bibliography[99], it seems that those patients with *EGFR* gene amplification analyzed by FISH are more likely to benefit from cetuximab therapy, so we have confirmed that DiFi cell line has amplification of *EGFR* gene.

a



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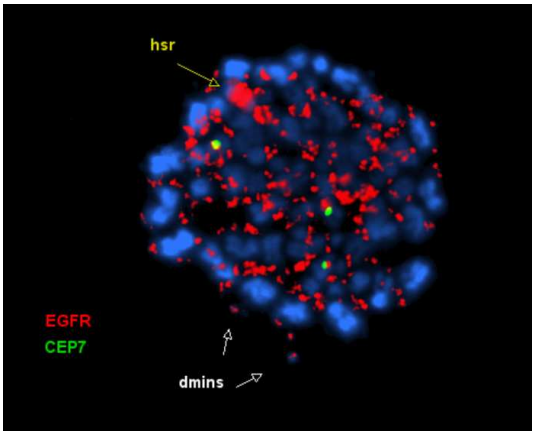


Figure R.3. DiFi cell line harbors *EGFR* gene amplification and overexpress the protein. a, WB analysis of total and P-EGFR in different CRC cell lines in basal conditions. Tubulin expression was used as loading control.**b,** FISH analysis of *EGFR* gene in DiFi cell line. An increase in *EGFR* gene copy number (in red) relative to chromosome 7 number (in green) is observed. Moreover, homogeneous staining regions (hsr, yellow arrows) and double minutes (ds, white arrows) chromosomes indicate amplification of the gene.

As shown in **Figure R.3.b**, DiFi cell line has more than 20 copies of *EGFR* gene per nuclei, which are clustered in homogeneously staining regions and double minutes chromosomes, two cytogenetic alterations that are described to occur as a result of oncogene addiction[167], suggesting that this cell line is addicted to EGFR signaling.
Moreover, direct sequencing of *KRAS, BRAF* and *PIK3CA* of DiFi DNA demonstrated that DiFi is WT for these genes (**Table R.1**).
So, as DiFi cell line is very sensitive to cetuximab and its genotype is similar as a patient that would typically respond to cetuximab, we have chosen this cell line to develop the model of cetuximab acquired resistance.

R.1.2. Acquisition of cetuximab resistant clones

DiFi cells were continuously exposed to cetuximab 1μg ml^{-1}, which is the minimal dose in which cetuximab achieve the maximum effect in this cell line, **Figure R.2.a**. Five months later, different DiFi-derived cetuximab resistant pools were established. In order to avoid genetic variability between different resistant cells we isolated single cell clones, which we called DiFi Cetuximab Resistant clones (DCR).

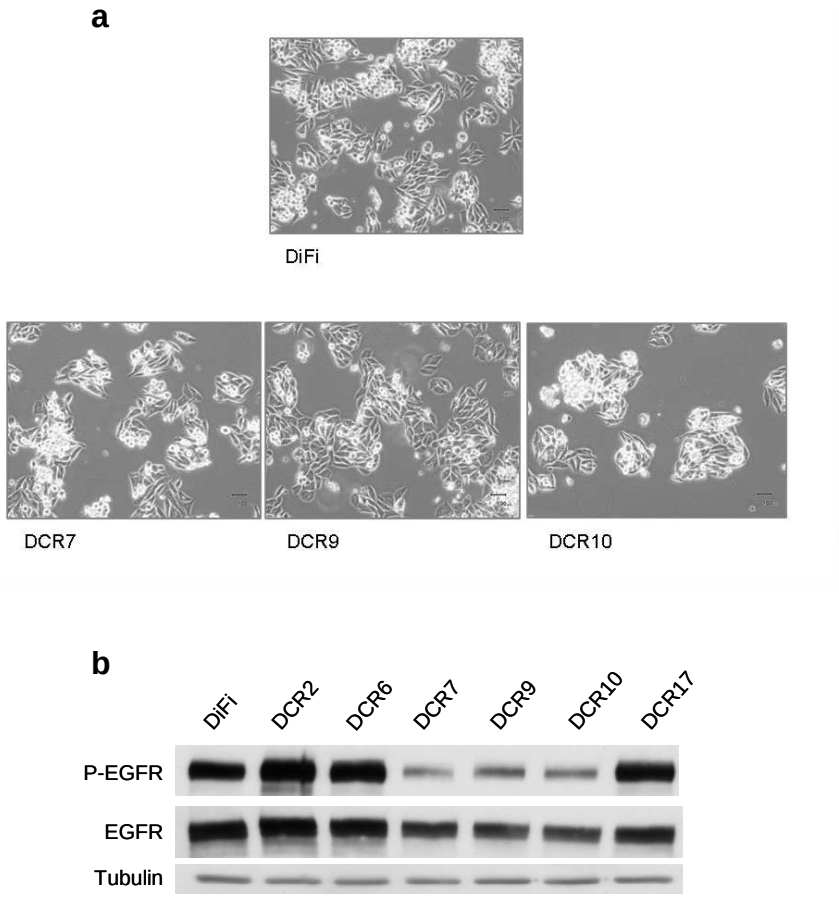


Figure R.4. Morphologically, DCR are indistinguishable from DiFi parental, but differ in EGFR expression levels. a, Representative images of DiFi and DCRs cells under light microscopy. **b,** WB analysis of P-EGFR and total EGFR in DiFi and different DCRs. Tubulin expression was used as loading control.

Under light microscopy, resistant cells exhibit a flat, epithelial morphology similar to the parental cells, suggesting that resistant cells had evolved from the sensitive cells, excluding any contamination (**Figure R.4a**). DCR7, 9 and 10 were chosen for further characterization, as they were coming from the same pool of resistant

cells and the three clones expressed lower levels of total and activated EGFR (**Figure R.4b**), suggesting that they would have acquired the same molecular mechanism of cetuximab resistance, as overactivation of an alternative receptor.

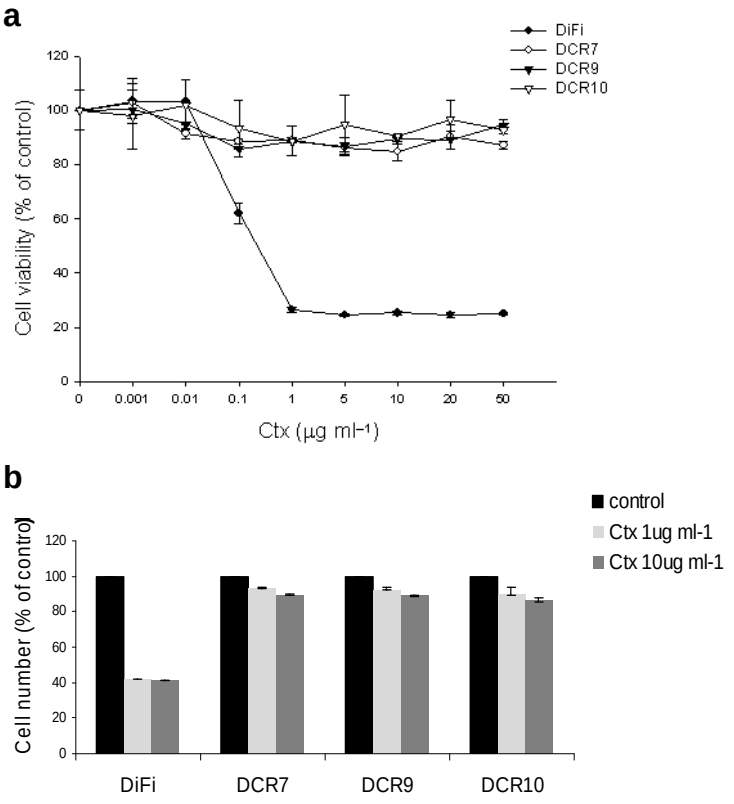
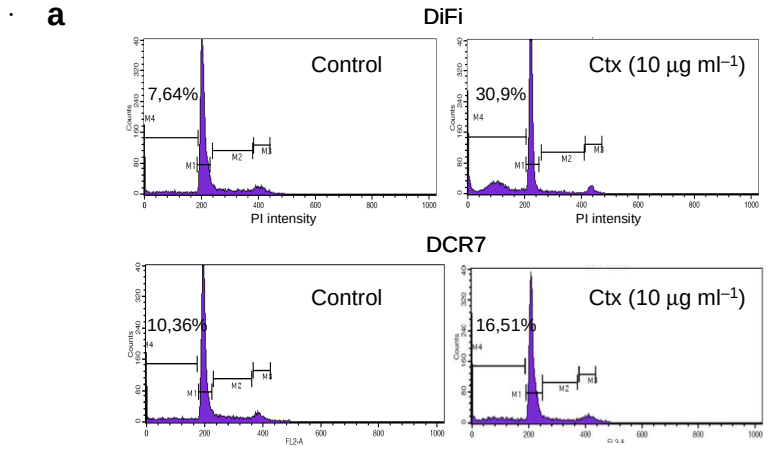


Figure R.5. DCRs are completely resistance to cetuximab treatment. a, MTS assay of DiFi and DCRs treated at the indicated concentrations of ctx for 48h. **b,** Crystal violet staining of DiFi and DCRs treated at 1 or 10 μg ml^{-1} of ctx for 48h. Error bars represent the standard deviation of three independent experiments.

By MTS assay we observed that all DCR were completely resistant to cetuximab treatment not only at the concentration in which they were obtained, 1μg ml^{-1}, but also at higher concentrations (**Figure R.5a**). We confirmed the results on cellular effects by crystal violet staining.

As show in **Figure R.5b**, while cetuximab treatment induced a decrease in DiFi cell number, no effects were observed in the three resistant clones



DiFi	G0/G1	S	M	subG0/G1
Control	72.56	10.73	6.68	7.64
Cetuximab	61.1	3.55	3.98	30.9

DCR7	G0/G1	S	M	subG0/G1
Control	69.26	11.7	6.46	10.36
Cetuximab	67.85	7.94	6.23	16.51

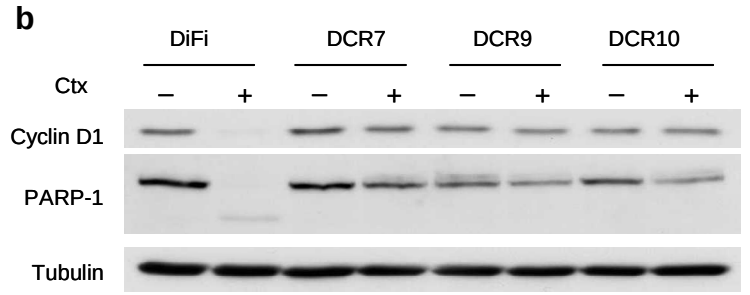


Figure R.6. Cetuximab (Ctx) resistant cells do not undergo cell cycle arrest or induction of apoptosis upon Ctx treatment. a, DiFi cells and DCR cells were treated with 10 µg ml-1 Ctx. After 24 hrs cells were harvested and subjected to cell cycle distribution analysis by flow cytometry. The histogram shows the percentage of cells in the subG$_0$/G$_1$ fraction and in each cell cycle phase. **b**, WB analysis of cyclin D1 expression and PARP cleavage in DiFi and DCR cells after treatment with 10 µg ml-1 Ctx for 24 hrs.

Consistently, cetuximab was unable to induce both cell growth arrest and apoptosis in the resistant clones, as compared to the parental cells. While in the parental cells an increase in the percentage of apoptotic cells (subG0/G1 fraction) as well as PARP cleavage and a decrease of cells in S and M phases as well as cyclin D1 abrogation were observed following cetuximab treatment, in the resistant clones none of these molecular events occurred (**Figure R.6a and R.6b**).

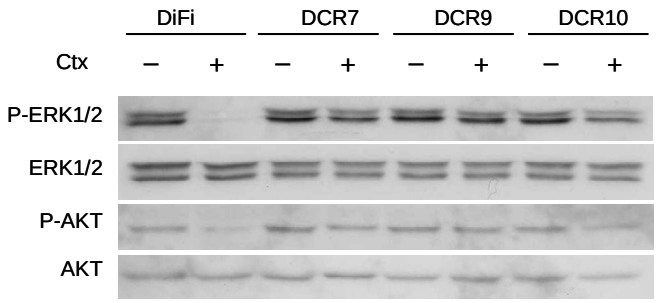


Figure R.7. Cetuximab is not able to inhibit EGFR dependent pathways in the DCRs. WB analysis of P-ERK1/2, ERK1/2, P-AKT and AKT expression in DiFi and DCRs treatmed with 10 μg ml-1 Ctx for 2 hrs.

Interestingly, unlike the parental DiFi cells, the activation of EGFR downstream effectors AKT and ERK was not suppressed by exposure to cetuximab. As observed in **Figure R.7**, while phosphorylated ERK1/2 and phosphorylated AKT were decreased upon two hours of cetuximab treatment in the parental cells, only a slight decrease in phosphorylated ERK1/2 was observed in the DCRs, indicating somehow that the resistant cells were able to maintain EGFR downstream pathways activated despite cetuximab exposure.

R.1.3. The molecular mechanism underlying the resistance persisted upon the time

Since we established DCRs, they have always been growing in the presence of cetuximab 1μg ml^{-1} in the media. We wondered if the mechanism underlying the resistance was transient and dependent of cetuximab exposure or was well established and independent of the antibody presence. The first hypothesis would indicate that resistant cells would be expressing something leading to the resistance but if we remove cetuximab from the media, the cells would recover its normal phenotype becoming again cetuximab sensitive cells. If, on the contrary, the mechanism is well established, as a genetic event, the resistance would persist upon time despite removing cetuximab from the media.

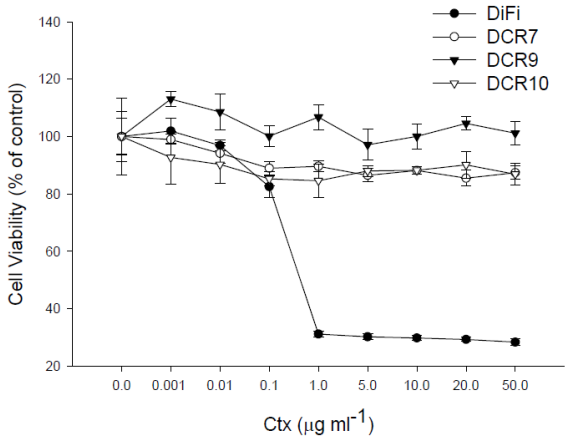


Figure R.8. DCRs remain resistant to cetuximab upon the time. MTS assay in DiFi and DCRs that were cultured for one month without the presence of cetuximab in the media after 48h of ctx treatment at the indicated concentration.

In order to answer this question, we cultured independently the three DCRs without the presence of cetuximab during 1 month. Then, cetuximab effects were assessed. As shown in **Figure R.8**, DCRs were still resistant to cetuximab, therefore the mechanism underlying cetuximab resistance was maintained upon the time despite removing cetuximab.

R.2. RESISTANT CELLS WERE DEPENDENT ON EGFR FOR THEIR GROWTH AND SURVIVAL

R.2.1. Cetuximab resistant cells did not acquire any previously described mutation conferring cetuximab resistance

The first question we wondered was if the DCRs had acquired under the selective pressure of cetuximab any of the previously reported mutations of cetuximab resistance. To answer this question we extracted DNA from the DCRs and sequenced *KRAS*, *B-RAF* and *PIK3CA* genes. As in DiFi cells, no mutations in these genes were found in the DCRs, suggesting that genetic alterations were not responsible of the molecular mechanism underlying cetuximab acquired resistance (**Table R.1**).

Table R.1. Mutational analysis of *KRAS, BRAF, PIK3CA* Exon 9 and 20 performed by direct sequencing in DiFi and DCRs.

	KRAS G12V	BRAF V600E	PIK3CA Ex9	PIK3CA Ex20
DiFi	WT	WT	WT	WT
DCR7	WT	WT	WT	WT
DCR9	WT	WT	WT	WT
DCR10	WT	WT	WT	WT

Apart from the assessed mutations which lead to aberrant activation of EGFR signaling pathways resulting in cetuximab resistance, PTEN loss of function can also lead with an increase of phophorylated AKT leading to resistance[70]; **Figure R9.a** shows that DiFi cells and all DCRs express similar levels of PTEN and no overactivation of AKT

was observed, suggesting that PTEN function was maintained in the resistant cells. Moreover, **Figure R9b** shows *EGFR* gene copy number in the DCRs, which despite expressing lower levels of EGFR protein as seen by WB they still harbor an *EGFR* gene amplification. Thus, considering classical issues that may affect cetuximab therapy, the cetuximab resistant clones have the same alterations as DiFi sensitive cells.

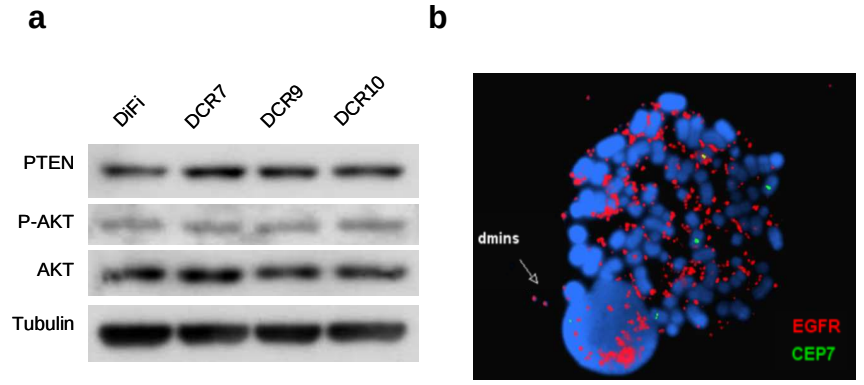


Figure R.9. DCRs present the same general alterations as DiFi parental cells. a, WB analysis of basal expression of PTEN, P-AKT and total AKT in DiFi and DCRs. Tubulin expression was determined as loading control. **b**, An increase in *EGFR* gene copy number (red) relative to chromosome 7 number (green) analysed by FISH in DCRs.

R.2.2. Cetuximab resistant clones were still dependent on EGFR signaling

DiFi cells are addicted to EGFR activation, as indicated by *EGFR* gene copy number amplification and their high sensitivity to anti-EGFR therapies as cetuximab. In other studies which try to understand the molecular mechanism of acquired resistance to anti-

EGFR therapies such as erlotinib or gefitinib in NSCLC, it has been observed that activation of other membrane TKR, as c-met, might be responsible of the underlying resistance[162]. Furthermore, in HNSCC cell lines which have also acquired resistance to cetuximab, different dimerization of HER family members restore the ability to activate EGFR downstream signaling pathways[164]. All theses previous results suggest that other RTKs may replace the addiction to EGFR conferring resistance to anti-EGFR therapies. To determine whether activation of an alternative RTK was mediating the resistance through ERK and AKT activation, we used a phospho-RTK array to compare the basal activation of 42 different RTKs in parental and resistant cells (**Figure R.10a**). Interestingly, while DiFi cells have an hyperactivation of many different RTKs, being EGFR the most activated, the resistant cells have less EGFR phosphorylation and much less phophorylation of other RTK, suggesting that no other RTKs were mediating ERK and AKT activation. The reason of the lower level of basal of TKRs phosphorylation in DCRs was not further addressed but probably, EGFR by heterodimerization was determining the phosphorylation status of other RTKs and as a consequence, as DCRs expressed less EGFR than DiFi, the status of other RTKs was lower.

To determine if the survival of the resistant clones was still mediated by EGFR, we pharmacologically inhibited EGFR activity in parental and cetuximab-resistant clones with the EGFR tyrosine kinase inhibitor gefitinib. We treated the cells during 48 hour at growing concentrations of gefitinib, and as observed in **Figure R.10b and R.10c**, DiFi parental cells and DCRs were equally sensitive to the compound. This sensitivity was correlated with the inhibition of EGFR downstream effectors ERK and AKT by WB analysis. All

these data suggests that DCRs remained dependent on EGFR for their growth and survival.

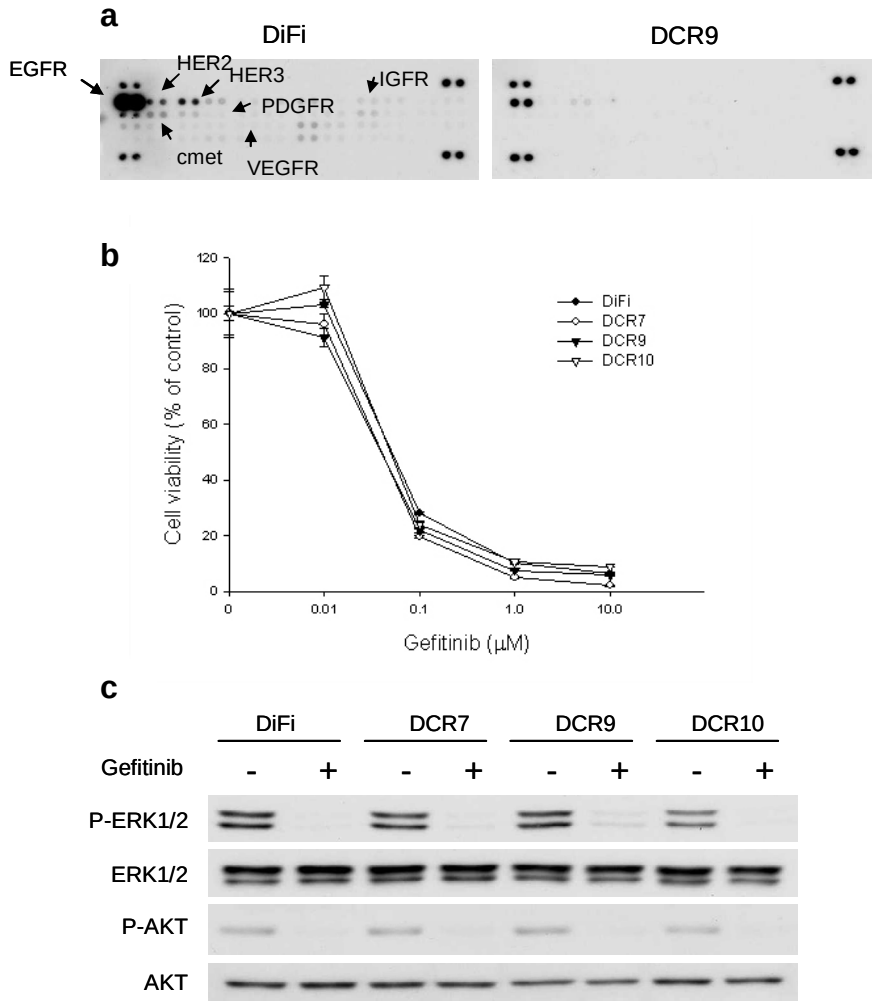


Figure R.10. DCRs are dependent on EGFR for their growth and survival. a, Cell lysates of DiFi and DCR9 in basal conditions were incubated with the RTK array. Each specific antibody is spotted twice in the membrane; therefore the signal of each phosphorylated-Receptor is seen as duplicates. Black arrows indicate the signal of specific RTKs that might be involved with cetuximab resistance. **b,** Parental and resistant cells are equally sensitive to gefitinib. Parental DiFi and DCR cells were treated with increasing doses of gefitinib and viable cells were measured by MTS assay after 48 hours. **c,** Sensitivity to gefitinib correlated with effective inhibition of EGFR downstream effectors (AKT and ERK). Cells were cultured for 2 hrs with gefitinib 100nM, cell lysates were subjected to WB analysis using antibodies against the indicated proteins.

R.3. PANITUMUMAB WAS EFFECTIVE IN CETUXIMAB SEVSITIVE AND RESISTANT CELLS

R.3.1. Cetuximab resistant cells were sensitive to panitumumab

We had shown that although cetuximab was not able to induce cell death neither decrease EGFR downstream signaling pathways in DCRs, they were still dependent on EGFR for their growth and survival.

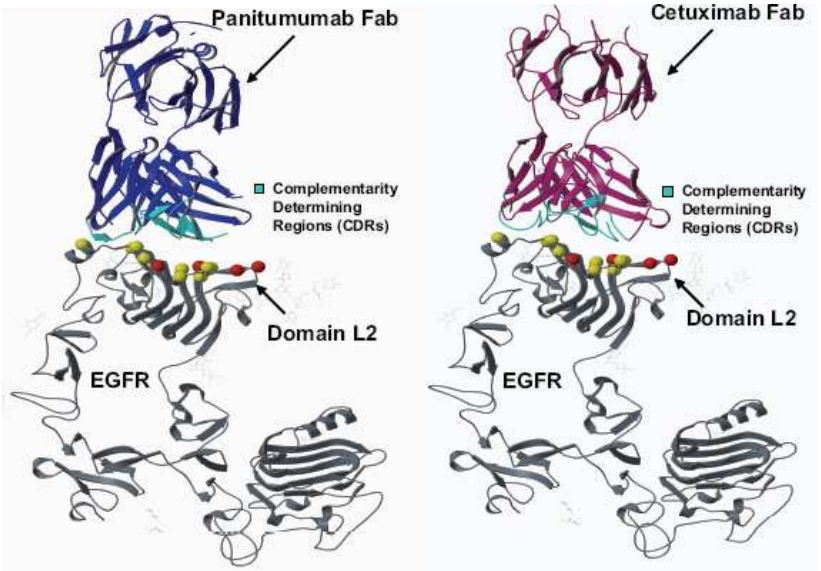


Figure R.11. Schematic representation of panitumumab Fab (blue) and cetuximab Fab (purple) binding epitopes on EGFR (grey). Red dots in EGFR domain III respresent the aminoacids critical for the interaction. Light blue areas of the antibodies represent the CDRs[168].

We then examined the effects of panitumumab, another anti-EGFR moAb that is currently approved for the treatment of colorectal cancer patients, on cetuximab-sensitive *versus* cetuximab-resistant cells.

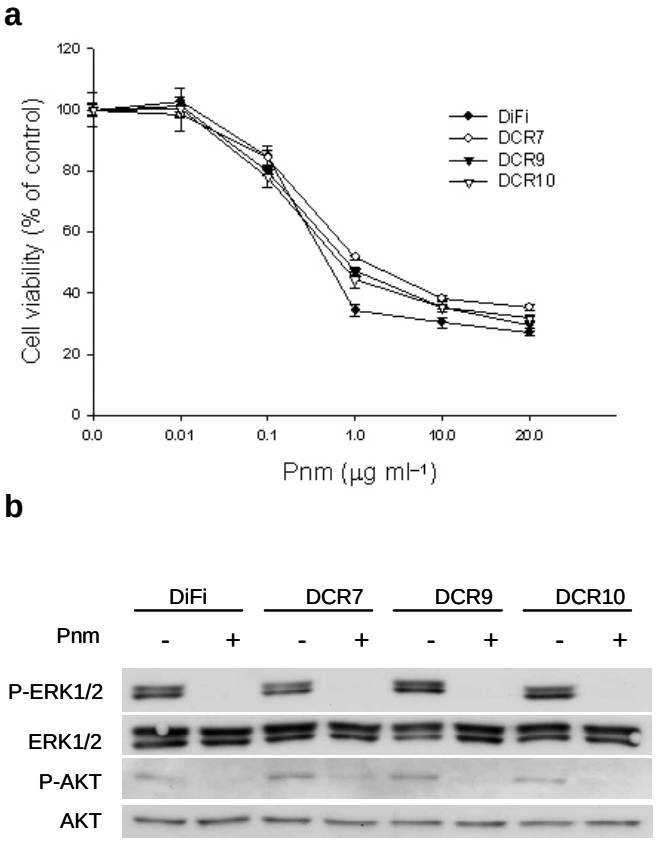
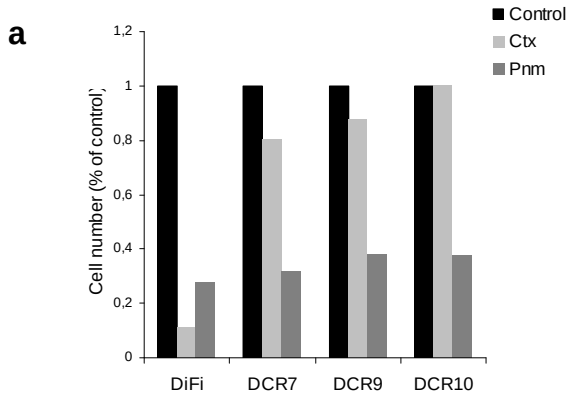


Figure R.12. Panitumumab (Pnm) decreased cell viability and EGFR downstram pathways in DCR cells. a, Parental DiFi and DCR cells were treated with increasing doses of Pnm during 48h, viable cells were measured by MTS assay **b,** Sensitivity to Pnm is correlated with effective inhibition of EGFR dowsntream effectors (AKT and ERK). Cell lysates from DiFi cells and DCRs were collected after treatment with Pnm 10μg ml^{-1} for 2hrs. WB analysis was performed using antibodies against the indicated proteins.

Panitumumab is a totally human IgG1 which has been described to recognize a similar epitope to cetuximab[168] (**Figure R.11**) preventing ligand binding, therefore both agents are supposed to have the same effects on EGFR inhibition. In fact, clinically are considered essentially similar. Surprisingly, cell viability of the DCRs was effectively inhibited by panitumumab. We treated DiFi and DCRs for 48h with panitumumab and by MTT assay we observed that while DCRs were totally resistant to cetuximab, they were equally sensitive as DiFi cells to panitumumab (**Figure R.12a**). As expected, these cellular effects correlated with the ability of panitumumab to inhibit EGFR downstream effectors ERK and AKT, as assayed by WB analysis of their phosphorylation (**Figure R.12b**). Manual counting and cyclin D1 expression and PARP1 cleavage analysis by WB of DiFi and DCRs treated with both antibodies confirmed that panitumumab effectively decreased cell cycle and induced apoptosis in the DCRs. **Figure R.13a and R.13b** show how panitumumab was able to decrease cyclin D1 expression and increase PARP1 cleavage while decreasing the number of both DiFi and DCRs.



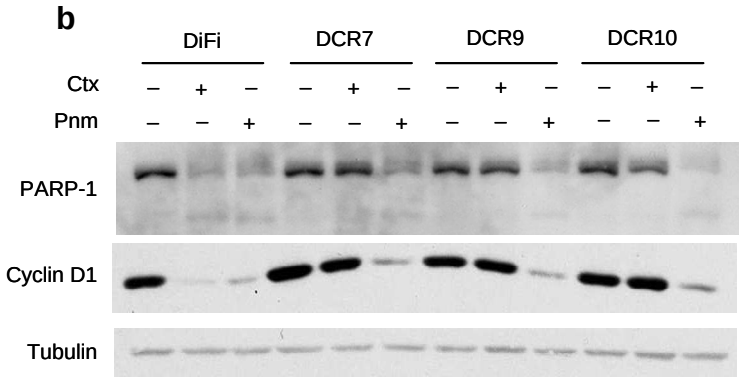


Figure R.13. Panitumumab (Pnm) induced apoptosis and growth arrest in DCR cells. a, Approximately 300,000 parental DiFi and DCR cells were plated in 6 well plates and were treated with Ctx or Pnm (10 μg mL-1). After 48 hours cells were trypsinized and viable cells were manually counted following trypan blue staining. **b**, WB analysis of cyclin D1 and PARP cleavage in DiFi and DCR cells treated with Ctx or Pnm (10 μg mL-1) for 24 hrs. Tubulin expression was used as loading control.

R.3.2. Panitumumab was able to prevent EGF stimulation of EGFR while cetuximab did not

Panitumumab and cetuximab are supposed to recognize the same region of EGFR and they bind to this region with more affinity than the natural ligands[169], **Figure R.14a.**

We first examined the ability of both antibodies to prevent the activation of EGFR by EGF. As observed in **Figure R.14b**, within 15 minutes of EGF stimulation there was an increase of EGFR phosphorylation in both sensitive and resistant cells. However, when we incubated the cells with each antibody prior to the addition of the ligand, panitumumab was able to prevent EGF stimulation in both

cetuximab-sensitive and -resistant cells, but cetuximab only disrupted EGF-mediated activation in the sensitive cells. These results suggested that the resistance mechanism in DCR cells was involving cetuximab binding to EGFR.

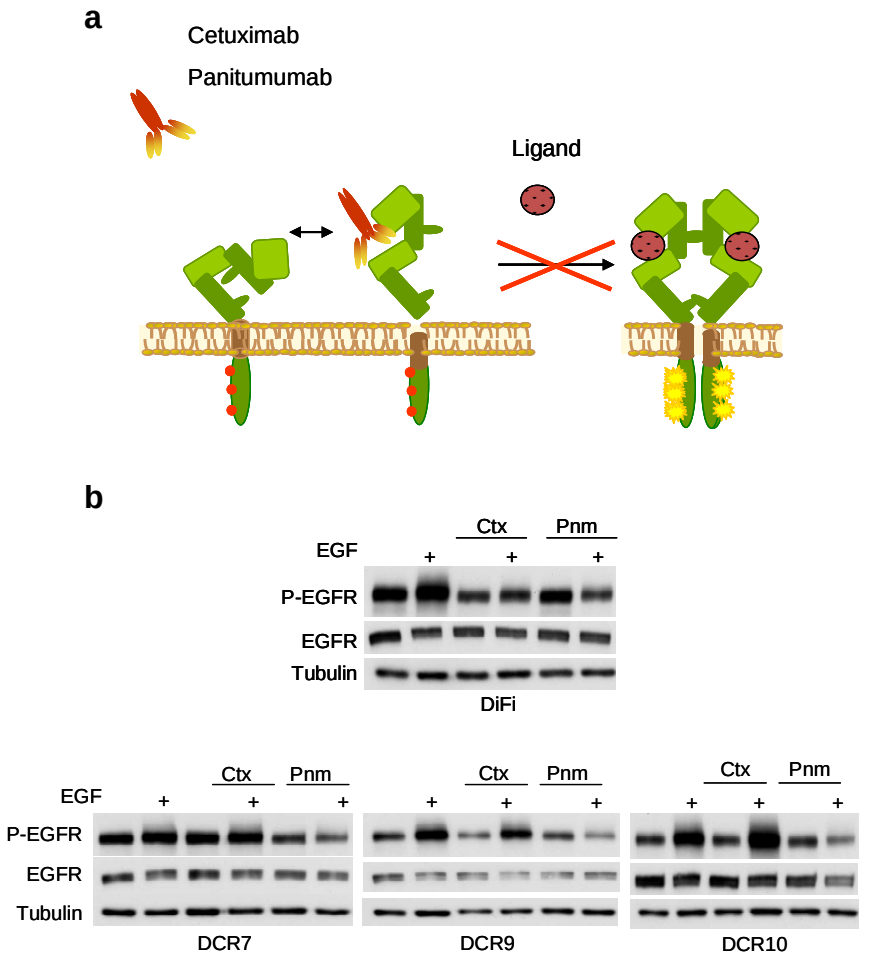


Figure R.14. Only panitumumab, but not cetuximab, was able to prevent ligand stimulation of EGFR.a, Schematic representation of ctx and pnm binding to EGFR preventing ligand binding. **b,** DiFi (upper panel) and DCRs (lower panels) were treated with Ctx and pnm 10 μg mL-1 for 2 hours prior to stimulation with EGF 10 ng mL-1. WB analysis of P-EGFR and total EGFR was determined. Tubulin expression was determined as loading control.

R.4. THE RESISTANT CELLS ACQUIRED A MISSENSE MUTATION ON *EGFR* UNDER THE SELECTIVE PRESSURE OF CETUXIMAB

R.4.1. Cetuximab resistant cells harbored a missense mutation in the extracellular domain of EGFR

Based on the unexpected activity of panitumumab in DCRs, we hypothesized that cetuximab resistance resulted from a change in the epitope of EGFR that cetuximab recognizes, which specifically affected the ability of cetuximab to prevent ligand effects on EGFR.
Thus, we decided to sequence *EGFR* gene coding region to identify if any mutation had occurred during the process of selection of DCR cells. Considering the magnitude of *EGFR* gene amplification and to avoid detecting mutations in unexpressed EGFR genes, we initially decided to sequence only EGFR mRNA.
As show in **Figure R.l5**, we designed 9 pair of primers to amplify overlapping DNA fragments of around 100bp which covered the entire EGFR coding sequence. We extracted RNA from DiFi cetuximab sensitive and resistant clones. After reverse transcription to cDNA, each EGFR fragment was amplified by PCR and sequenced by direct sequencing. Interestingly, a new mutation, an A→C substitution at nucleotide 1476 was detected in each of the three DCRs but not in the cetuximab-sensitive DiFi cells (**Figure R.16**).

Figure R.15. Schematic view of the primers designed to amplify and sequence all EGFR coding sequence. The sequence observed is EGFR mRNA transcript variant 1 sequence (NM_005228.3), grey background indicates the coding sequence. The 9 overlapping pairs of primers are represented in specific colors for each pair.

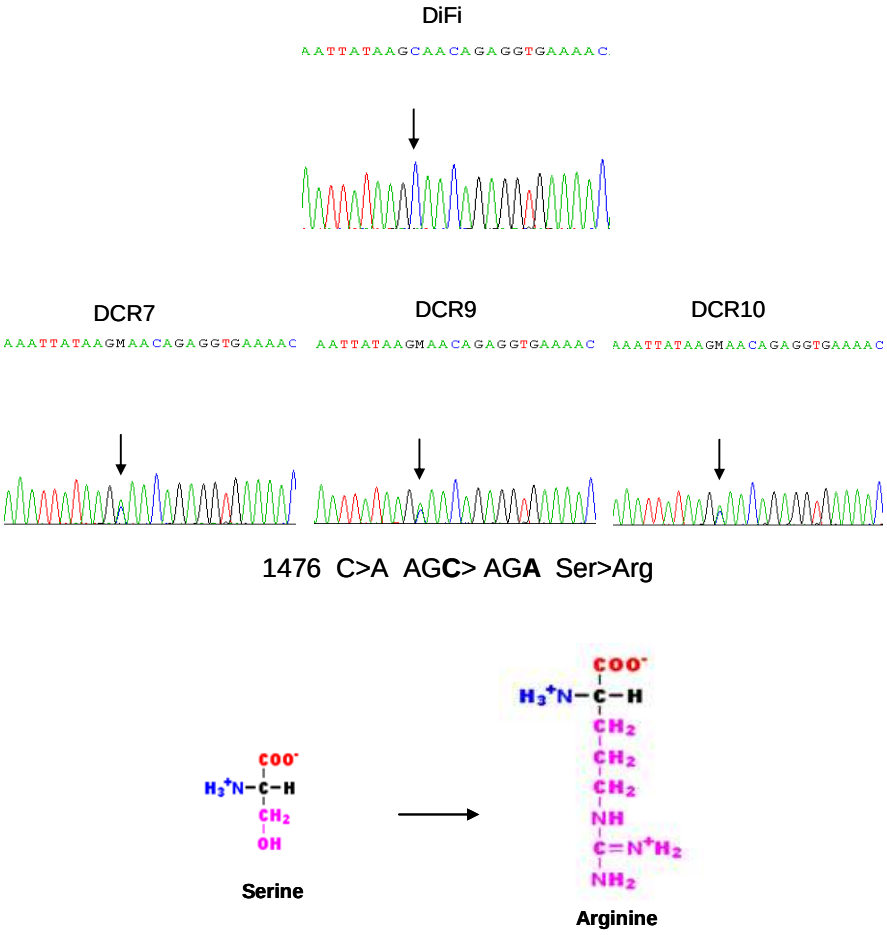


Figure R.16. DCRs had acquired a missense mutation under the selective pressure of cetuximab. Total RNA was extracted from DiFi and DCRs and all EGFR cds was sequenced. The upper panels show a heterozygous mutation (arrows) present in DCRs that is not present in DiFi parentals cells. The mutation is in position 1476 and results in C>A change that codifies for Arginine instead of Serine. Lower panel shows Serine and Arginine; each amino group is represented in blue, the acid group in red and the specific chain in pink.

There were no other acquired alterations detected in the *EGFR* coding sequences. The 1476 A→C change results in the substitution of a serine to an arginine at the position corresponding to amino acid 492

of the human EGFR protein. **Figure R.17** shows EGFR different domains within the coding sequence and as observed, 1476 A→C change is located within the extracellular domain of EGFR (domain III), which is the domain were cetuximab epitope is located. Serine and Arginine differ in charge and size (**Figure R.16**), therefore this aminoacid change might be relevant for cetuximab interaction with the receptor.

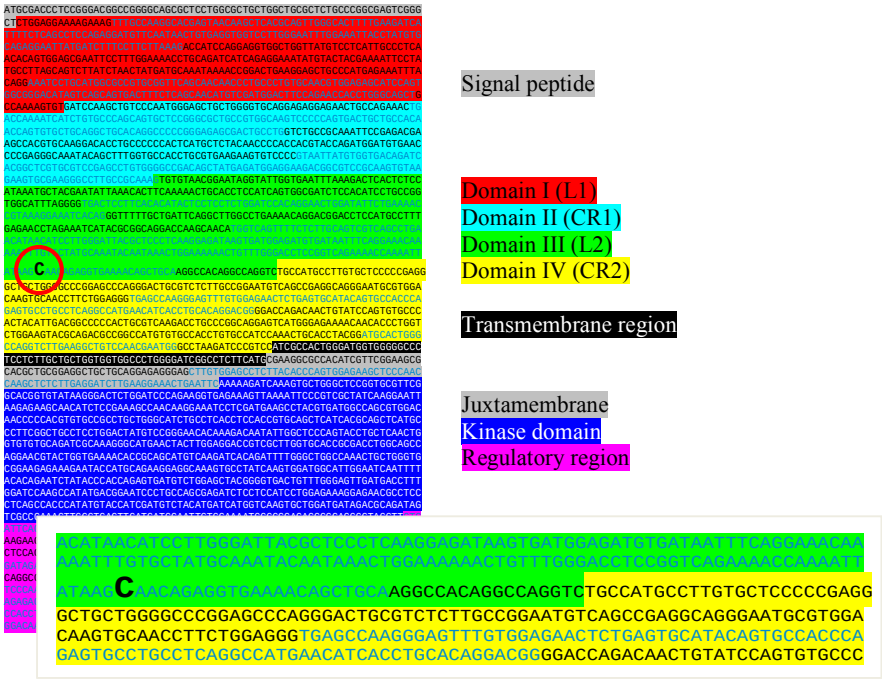


Figure R.17. EGFR 1476 C>A mutation is located in domain III of EGFR cds. Schematic representation of 1476C>A mutation within EGFR cds. The sequences that codify to each specific domain are shown in different colors, Domain III (green) codifies for position 1476 (red circle).

R.5. ROLE OF EGFRS492R ON CETUXIMAB TREATMENT

R.5.1. Establishment of EGFRWT and EGFRS492R expressing cells

DiFi cells and DCRs had been cultured as independent cultures for more than five months so many differences might have appeared in both cell lines independently of the differences leading to cetuximab resistance. In order to validate the new found EGFR mutation as the acquired molecular mechanism responsible of cetuximab resistance, we needed to express EGFRWT and EGFRS492R in the same cellular model with the same background in order to exclusively compare the effects of the mutation. For that reason, we decided to express full-length EGFRWT and the EGFRS492R in cultured mouse embryonic fibroblast NIH3T3. We chose NIH3T3 cell line because it lacks detectable endogenous EGFR protein levels and because cetuximab or panitumumab are not able to recognize murine EGFR. We obtained EGFR cDNA cloned into pBABEpuro lentiviral vector. By site directed mutagenesis we introduced C1722A mutation in EGFR cDNA, and we transfected both plasmids into HEK293T derived phoenix cells to produce lentiviral particles. NIH3T3 cells were infected with each viral supernatant and after puromicyn selection RNA was sequenced and WB was performed to see EGFR expression and the desired mutation in the newly established cell lines. **Figure R18** shows effective expression of EGFRWT and EGFRS492R in NIH-3T3 cells.

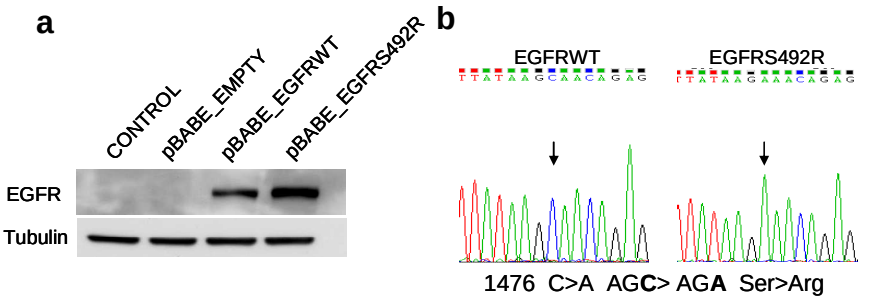


Figure R.18. EGFRWT and EGFRS492R are correctly expressed in NIH3T3 cells. a,Vector (pBABE) containing the cds of WT EGFR (pBABE_EGFRWT) or S492R mutant EGFR (pBABE_EGFRS492R) were retrovirally infected into NIH3T3 cell to overexpress WT EGFR or S492R EGFR proteins, respectively. NIH3T3 cells infected with the empty vector (pBABE_EMPTY) were used as control and non infected NIH3T3 cells were used as negative control. **b,** RNA was extracted from NIH3T3 infected with pBABE_EGFRWT and pBABE_EGFRS492R, arrows indicate the nucleotide in which the mutation (C>A) is localized by direct sequencing.

R.5.2. EGFR mutation impaired cetuximab effects on preventing ligand stimulation

The first question we asked was if cetuximab was able to impair ligand stimulation of the mutant receptor.

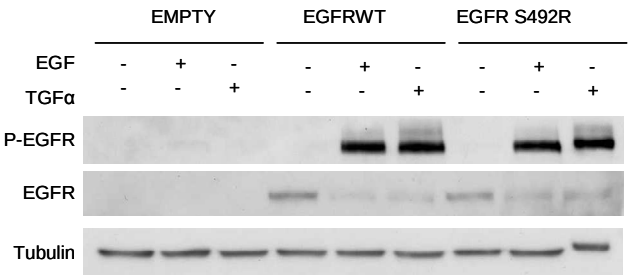


Figure R.19. EGF and TGFα stimulated EGFR in EGFRWT and EGFFS492R expressing cells. NIH3T3 cell expressing EGFRWT and EGFRS492R were stimulated with EGF and TGFα 10 ng ml-1 for 15 minutes. Cell lysates were subjected to WB analysis of total and phosphorylated EGFR. Tubulin expression was used as loading control. NIH3T3 infected with the empty vector (EMPTY) were used as negative control.

We stimulated NIH3T3 cells with EGF and TGFα, the classical EGFR ligands. As observed in **Figure R19**, in both EGFRWT and EGFRS492R expressing cells an increase of EGFR phosphorylation and a decrease of total EGFR protein expression was induced after ligand stimulation, however in NIH3T3 expressing the empty vector EGFR phosphorylation was not detected, confirming that the results observed were due to the ectopically infected EGFR.

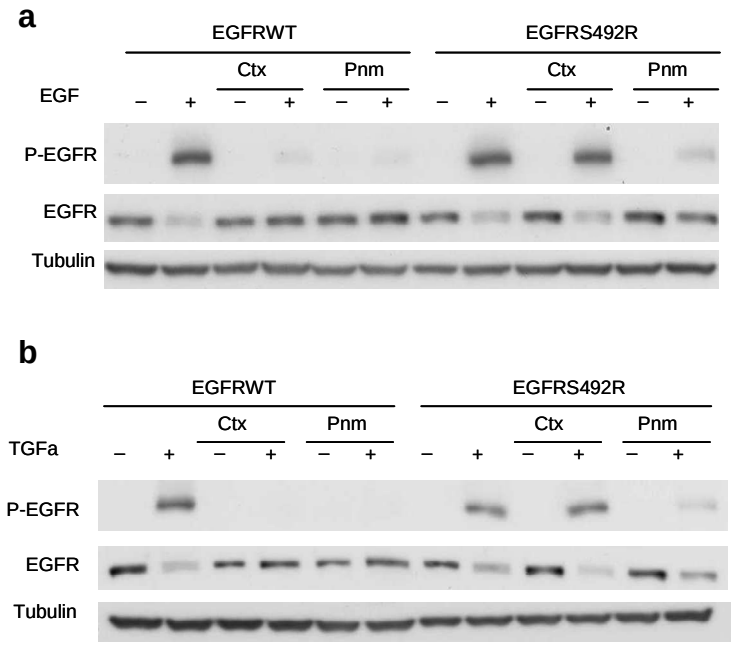
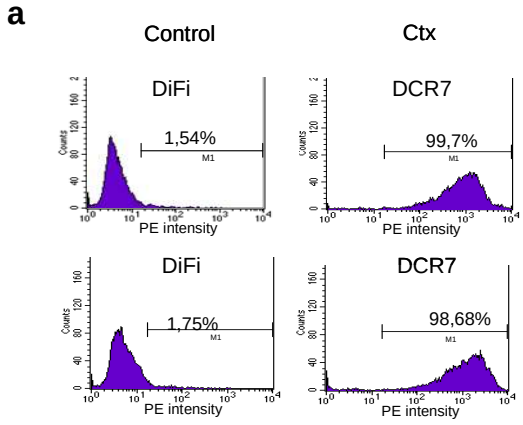


Figure R.20. Cetuximab is not able to prevent EGFRS492R stimulation of EGFR ligands, while panitumumab does. NIH3T3 cell expressing the EGFRWT and EGFRS492R were treated with ctx and pnm 10μg ml-1 for 2 hours. Then, cells were stimulated with EGF (**a**) and TGFα (**b**) 10 ng ml-1 for 15 minutes. Cell lysates were subjected to WB analysis of total and phosphorylated EGFR. Tubulin expression was used as loading control.

Then, we incubated both cell lines with cetuximab or panitumumab prior to ligand stimulation. **Figures R20a** and **R20b** show how in EGFRWT cells, both cetuximab and panitumumab inhibited EGFR activation by EGF and TGFα, whereas in EGFRS492R expressing cells, panitumumab, but not cetuximab, effectively blocked EGF-induced EGFR activation.

R.5.3. Cetuximab was not able to recognize and bind mutant EGFR

We had observed that the EGFRS492R mutation was responsible of cetuximab inability in preventing ligand stimulation. The next question we asked was if the mutation was impairing cetuximab recognition and binding of its epitope on EGFR. Flow cytometry analysis of antibody binding in NIH3T3 EGFR expressing cells showed that while cetuximab and panitumumab were able to interact with approximately 60% of cells expressing EGFRWT, only panitumumab was able to bind to cells expressing the EGFRS492R **(Figure R.21b)**.



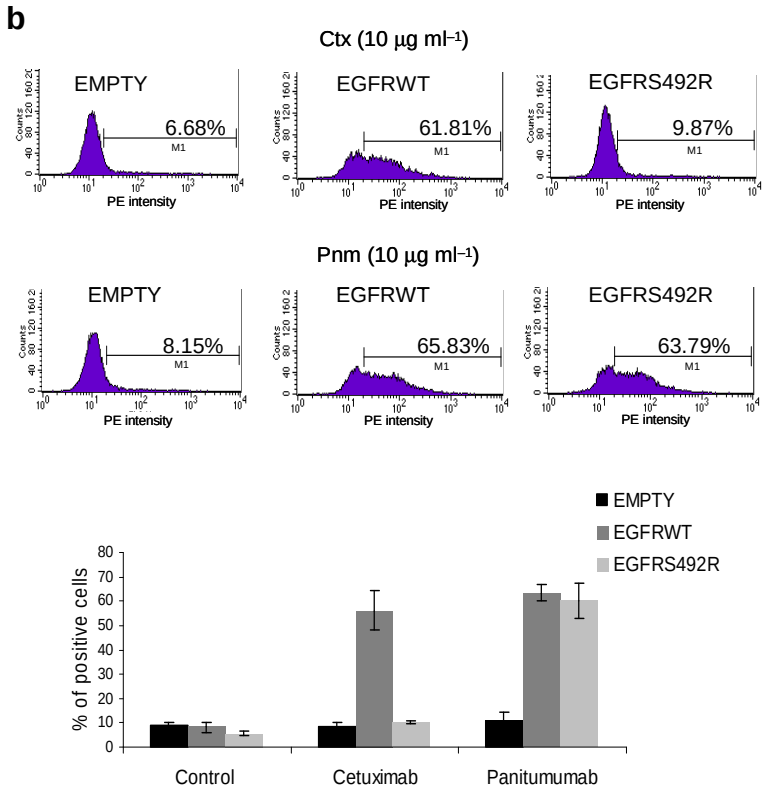


Figure R.21. Only Panitumumab, but not cetuximab, is able to bind EGFRS492R. a, DiFi in DCRs were harvested by tripsinization and incubated with cetuximab 10 µg ml^{-1} . A goat anti-human IgG conjugated with phycoeritrin was used as secondary antibody. Histograms indicate the percentage of cells positive or negative for phycoeritrin signal, which represents cetuximab binding. **b**,NIH3T3 cell expressing the EGFRWT and EGFRS492R were incubated with ctx and pnm 100ng ml^{-1} and antibody binding to EGFR was analyzed by flow cytometry using a secondary antibody to human IgG conjugated with PE. NIH3T3 cells expressing the empty vector (EMPTY) were used as negative control. Upper panel show the histograms obtained in the flow cytometry, graph in lower panel shows the median results of three independent experiments.

Notably, cetuximab was able to identify DCRs cells with high efficiency, similar to parental cells (**Figure R.21a**). This observation confirms the heterozygous nature of the EGFR mutation in DCRs

cells, which express both mutant and WT EGFR which still can be detected by cetuximab.

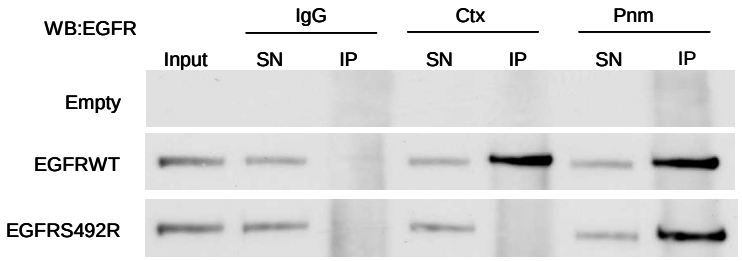


Figure R.22. Only Panitumumab, but not cetuximab, was able to bind EGFRS492R. Cell lysates of NIH3T3 cells expressing the EGFRWT and EGFRS492R were incubated with ctx, pnm and an irrelevant IgG at 100ng ml^{-1}. Protein A/G sepharose beads were used to precipitate the antibodies. The complete cell lysate (Input), the supernatant fraction (SN) and the immunoprecipitated fraction (IP) were subjected to WB analysis of total EGFR. NIH3T3 cells expressing the empty vector (EMPTY) were used as negative control.

To confirm these results we immunoprecipitated EGFR from cell lysates of EGFRWT and S492R expressing cells using cetuximab and panitumumab. Both cetuximab and panitumumab could bind to and precipitate EGFR in WT EGFR cells; however, in cells expressing EGFRS492R, EGFR was detected in panitumumab immunoprecipitated fraction but not in cetuximab immunoprecipitated fraction (**Figure R22**).

To further verify that the S492R mutation on EGFR directly impacted the binding to cetuximab *in vitro*, we produced EGFRWT and S492R ectodomains fused with human Fc recombinant proteins (ECD-Fc). The recombinant proteins were attached in plastic plates and antibody binding of the proteins was assessed by ELISA. First we assessed the direct binding of each antibody, cetuximab and panitumumab, to WT

ECD and S492R ECD. As shown in **Figure R.23a**, while panitumumab could bind to both ECD, cetuximab only bound to WT ECD. Moreover, we assessed how both antibodies competed for the binding to each ECD. We incubated both plates with a fixed concentration of biotin-labeled panitumumab, which could be detected by SA-HRP, and then we added serial dilutions of panitumumab, cetuximab, and a control IgG, to test how each antibody could replace the binding of biotin-labeled panitumumab to WT and S492R ECD.

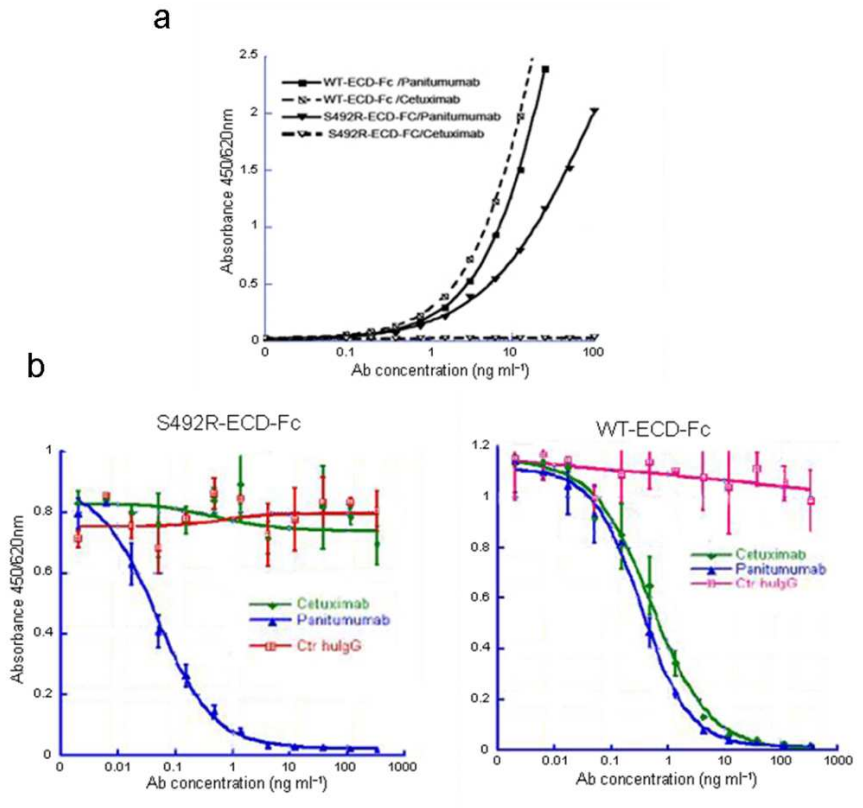


Figure R.25. Only Panitumumab, but not cetuximab, was able to bind EGFRS492R ectodomain. **a,** we compared the ability of ctx and pnm to interact with WT and S492R EGFR ECD by direct binding assay and **b,** the ability of both antibodies to compete with pnm by competitive binding assay. Human IgG was used as negative control of the binding.

As shown in **Figure R.23b** both antibodies compete for the binding to WT ECD, but only panitumumab could compete for the binding to S492R ECD. Consistent with the results obtained in cell lines the binding by ELISA confirmed that the S492R EGFR mutant was selectively defective for binding to cetuximab, but not to panitumumab.

Collectively, these data indicated that the S492R missense mutation in EGFR contributes to acquire resistance to cetuximab by preventing binding of cetuximab to its epitope within the extracellular domain of EGFR. However, this amino acid change did not affect the ability of panitumumab to bind to EGFR, and panitumumab effectively suppressed EGFR signaling in these cells.

R.6. ROLE OF EGFRS492R IN THE CLINICAL SETTING

R.6.1. EGFRS492R mutation was detected in patients with acquired resistance to cetuximab

To assess the clinical relevance of this mutation as a mechanism of acquired resistance to cetuximab, we examined whether the S492R EGFR mutation could be found in colorectal cancer patients who experienced disease progression following an initial response to cetuximab. We analyzed paired tumor samples from patients before receiving cetuximab therapy (tumor specimen obtained for diagnosis) and after failure to cetuximab treatment (post-treatment specimen).

All pre-treatment samples were obtained from the primary colorectal tumor except in the case of patient number 3 were the specimen was obtained from a metastatic liver lesion. Post-treatment samples were from liver metastasis obtained by percutaneous biopsy with ultrasound guidance, except sample from patient 9 that was obtained from the primary colon tumor. There were no biopsy-related complications.

Table R.2 describes the clinical characteristics of ten patients for whom biopsy was obtained following progression on cetuximab-based therapy. Six of the patients had previously received at least one line of chemotherapy for the treatment of colorectal metastatic disease.

The mutational status of the *EGFR* as well as *KRAS*, *BRAF* and *PIK3CA* were assessed by DNA Sanger sequencing in all samples.

Table R.2. Treatment characteristics of patients that acquired resistance to cetuximab.

Patient Nº.	Treatment regimens for metastatic disease prior to cetuximab	Cetuximab regimen	Cetuximab dose intensity	Best response to cetuximab	Duration of response to cetuximab (weeks)
1	FOLFOX-4	FOLFIRI+cetuximab	>90%	SD	23
2		mFOLFOX-6+cetuximab	>90%	PR	64
3		mFOLFOX-6+cetuximab	>90%	PR	45
4	FOLFOX-4, FOLFIRI	irinotecan+cetuximab	≈80	PR	46
5	mFOLFOX-6, FOLFIRI	irinotecan+cetuximab	≈80	PR	52
6	FOLFOX-4	irinotecan+cetuximab	>90%	PR	27
7	mFOLFOX-6, FOLFIRI	irinotecan+cetuximab	>90%	PR	38
8	CapeOX	irinotecan+cetuximab	>90%	PR	20
9		FOLFOX-4+cetuximab	>90%	SD	42
10		FOLFIRI+cetuximab	>90%	PR	32

Table R.3. Clinical and mutational characteristics of metastatic colorectal cancer patients with paired biopsies before and after receiving treatment with cetuximab.

			Pre-cetuximab status					Post-cetuximab status				
Patient Nº.	Sex	Age	*KRAS*	*BRAF*	*PIK3CA*	EGFR gene copy number	*EGFR*	*KRAS*	*BRAF*	*PIK3CA*	EGFR gene copy number	*EGFR*
1	M	55	WT	WT	WT	WT	WT	G12V	WT	WT		WT
2	F	42	WT	WT	WT	WT	WT	WT	WT	WT		WT
3	M	64	WT	WT	WT	amplif	WT	WT	WT	WT	amplif	**S492R**
4	F	54	WT	WT	WT	WT	WT	WT	WT	WT		WT
5	M	59	WT	WT	WT	WT	WT	WT	WT	WT	amplif	WT
6	M	54	WT	WT	WT	WT	WT	WT	WT	WT	amplif	WT
7	M	62	WT	WT	WT	WT	WT	WT	WT	V600E		WT
8	M	79	WT	WT	WT	WT	WT	WT	WT	WT		WT
9	M	52	WT	V600E	WT	amplif	WT	WT	WT	V600E	amplif	**S492R**
10	M	61	WT	WT	WT	WT	WT	WT	WT	WT		WT

.

Quantitative RT-PCR to specifically detect the 1476 A→C EGFR mutation was also performed in all samples. The S492R EGFR mutation was also assayed by next generation high-throughput or deep sequencing (Genome Analyzer IIx, Illumina). In addition, EGFR gene copy number was studied by FISH in all specimens.

As shown in **Table R.3**, all pre-treatment biopsies were wild-type for *EGFR*, *KRAS*, *BRAF* and *PIK3CA*, except patient 9 that harbored a V600E *BRAF* mutation. Notably, among the post-cetuximab biopsies we identified the S492R mutation in two cases (patient 3 and patient 9). While patient 9 harbored the same nucleotide change as the one detected in the cell culture model (1476 C→T), the observed mutation in patient 3 was associated with a different nucleotide substitution affecting the same codon. This patient's tumor was found to harbor an A→C change at nucleotide 1474, which also results in a serine to arginine substitution at amino acid 492 of EGFR (**Figure R24 and Table R.3**).

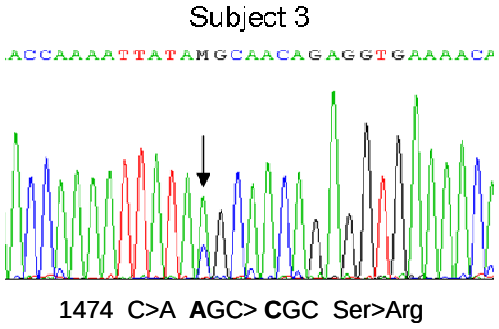


Figure R.24. Nucleotide sequence of the *EGFR* gene in tumor specimen from patient 3 at the time of progression to cetuximab. Direct sequencing analysis denotes a heterozygous mutation at position 492 encoding an Arginine instead of a Serine (arrow).

Of note, the S492R mutation in patient 9 was detected by high-throughput analysis (at a frequency of 3%) as well as by qPCR, but not by Sanger sequencing analysis, probably due to the lower sensitivity of this technique.

Deep sequencing of DNA sample from patient 3 confirmed the mutation in 25% of the cells. Sequencing of histologically normal colon tissue adjacent to the tumor from patient 3 and 9 showed only the wild-type sequence, indicating that the S492R mutation was a somatic mutation. To examine whether this mutation was already present in pre-treatment samples of these patients and had arisen secondary to drug selection, deep sequencing of pre-treatment tumor specimens from both patients was performed, which did not detect the mutation. Interestingly, both patients harboring the S492R mutations also demonstrated *EGFR* gene amplification (in both pre- and post-cetuximab specimens) as seen by FISH analysis (**Figure R.25**).

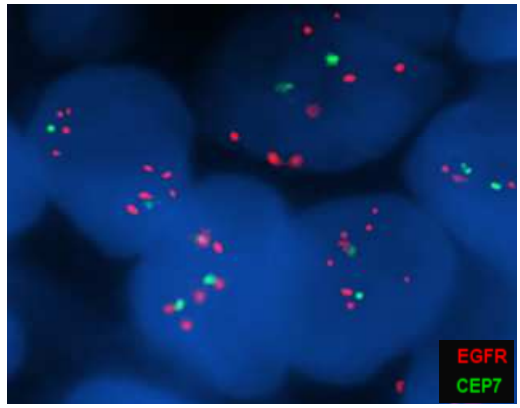


Figure R.25. FISH analysis of tumor specimen of subject 3. An increase in *EGFR* gene copy number (in red) relative to chromosome 7 number (in green) is observed, reflecting an amplification of the gene.

Post-cetuximab tumor sample from patient 3 did not harbor known *KRAS*, *BRAF* or *PIK3CA* mutations. Post-cetuximab tumor sample from patient 9 harbored the V600EBRAF mutation that was already observed in the pre-treatment specimen when the tumor was not resistant to cetuximab.

Significantly, among the other six patients, four demonstrated molecular alterations in components of the EGFR pathway in their post-cetuximab tumor specimen that were not present before treatment, potentially accounting for the acquisition of resistance. Thus, post-treatment tumor sample from patient 1 harbored a *KRAS* mutation, tumor specimen from patient 7 exhibited a *BRAF* mutation and tumor samples from patients 5 and 6 displayed EGFR gene amplification (**Table R.3**).

R.6.2. The S492R EGFR mutation and primary resistance to cetuximab

Mutations that contribute to secondary treatment resistance can also be responsible for the initial lack of response to a drug (primary or *de novo* resistance)[170,171]. To determine whether the S492R EGFR mutation could also potentially contribute to primary resistance to cetuximab, we sequenced the *EGFR* extracellular domain and analyzed *KRAS* and *BRAF* mutational status in primary tumor specimens from 83 metastatic colorectal cancer patients prior to administration of cetuximab-based therapy. Notably the S492R mutation was not found in any of the patients by directed Sanger sequencing and RT-PCR for the 1476 A→C EGFR mutation. The

lack of detected S492R mutation in untreated colorectal cancer patients was confirmed in a second unrelated set of 73 tumor samples from colorectal cancer patients from the U.S.A.

R.6.3. Response to panitumumab after cetuximab failure in a patient harboring the S492R mutation

Patient 3, whose post-treatment tumor harbored the EGFR S492 mutation, was a 64-year-old man with primary colon carcinoma and multiple non- resectable lesions in the liver. The tumor-node-metastasis (TNM) clinical stage was designated stage IV. The tumor did not harbor any known *KRAS* mutation and the patient was treated with cetuximab (400 mg/m^2 initial dose followed by 250 mg/m^2/week thereafter) plus FOLFOX-4 (oxaliplatin 85 mg/m^2 on day 1, plus leucovorin 200 mg/m^2 and fluorouracil as a 400 mg/m^2 bolus followed by a 600 mg/m^2 infusion during 22 hours on days 1 and 2)[113]. Three months after onset of treatment, a CT scan showed a partial response according to the response evaluation criteria in solid tumors (RECIST)[172]. After 10 months of treatment, however, hepatic lesions exhibited frank progression and new liver lesions appeared. Cetuximab treatment was discontinued and a biopsy from preexisting liver lesion was then obtained for molecular analysis, revealing the S492R EGFR mutation. Patient 3 was then treated with irinotecan-based chemotherapy plus antiangiogenic therapy (bevacizumab) but did not respond. At that point, therapy with single agent panitumumab (6mg/Kg every 2 weeks) was then initiated in this patient, and after two months of treatment, a CT scan showed a reduction in all liver lesions greater than 50% (**Figure R.26**) with a marked decline in the

carcinoembrionic antigen (CEA) blood tumor marker. After five month of treatment with panitumumab, subject 3 showed disease progression.

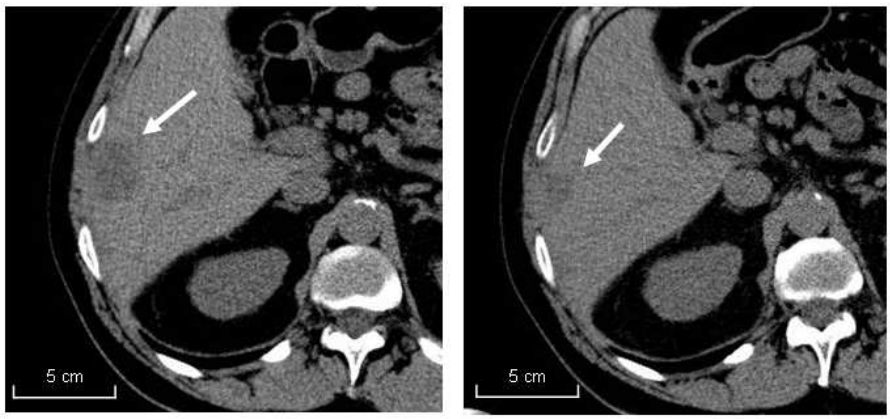


Figure R.26. A tumor that acquired EGFRS492R mutation responded to panitumumab after cetuximab failure. Computed tomographic (CT) scan of target metastatic lesion in the liver (segment VI) from the same patient, before panitumumab therapy was begun (left) and after 2 months of panitumumab treatment (right). White arrows mark the large volume of the lesion and its decrease after treatment (60% decrease).

The other patient that harbored the S492R EGFR mutation after progression to cetuximab (patient 9) was not alive when the EGFR analysis showing the S492R mutation was performed.

DISCUSSION

Identification of a mutation in the extracellular domain of EGFR conferring cetuximab resistance in mCRC

The development of cetuximab therapy represents a milestone in the treatment of colorectal cancer, yet its activity is limited to a subset of patients and its benefit is temporary. While effort is being done to identify the population that is more likely to benefit from the treatment, the understanding of the acquisition of resistance remains a challenge in the clinical practice. Once a patient progresses to cetuximab therapy, no more anti-EGFR approaches, such as the use of another anti-EGFR antibody (i.e. panitumumab), are considered. By an *in vitro* model of acquired resistance to cetuximab we have addressed this issue.

Clinical studies suggest that patients who are more likely to benefit from cetuximab therapy harbor *EGFR* gene amplification analyzed by FISH. Furthermore, tumors should not present activating mutations in *KRAS, BRAF* and *PIK3CA* gene neither PTEN loss of function. From all of these markers, the only one that is considered to be validated and therefore has clinical utility to date is *KRAS* mutation, which excludes patients from receiving anti-EGFR moAbs. We have exposed a CRC cell line highly sensitive to cetuximab, which has the optimum genetic background to respond to the therapy (i.e. *EGFR* gene amplification and no known mutations of primary resistance), with the antibody during several months. By chronic exposure to cetuximab, we have obtained different clones completely resistant. Cetuximab was no longer stopping the cell cycle or causing cell death in the resistant cells, moreover EGFR downstream signaling cascades were maintained despite cetuximab exposure. Interestingly, the genetic

background considering the classical markers of cetuximab resistance was not altered in these cells and they remained dependent on EGFR signaling for their growth and survival, being sensitive to EGFR TK inhibitors such as gefitinib. Unexpectedly, cetuximab resistant cells responded to panitumumab treatment, which effectively induced cell death and prevented EGF stimulation of EGFR. We identified in the cetuximab resistant cells a mutation affecting the extracellular domain of EGFR, that we termed EGFRS492R, which was not present in the parental cells. Serine and arginine differ in size and charge; while serine is small and neutral, arginine is a big basic aminoacid, and therefore this single aminoacid change may affect cetuximab interaction. The presence of this genetic event would explain why the resistant cells remain resistant long time after removing cetuximab from the media. We have analyzed the specific role of the EGFRS492R mutation in NIH3T3 cells, which are from mouse origin and lack endogenous EGFR expression. Notably, we have demonstrated by different approaches that cetuximab is not able to bind to EGFRS492R. However, the nature of the observed amino acid change within the extracellular domain of EGFR allows it to retain panitumumab-binding capacity *in vitro*, suggesting distinct EGFR binding by these two antibodies that are differentially impacted by the observed mutation. The mutation was heterozygous in cetuximab resistant cells, indicating that WT and mutated EGFR were coexpressed. This observation may explain the results obtained by flow cytometry analysis were cetuximab is able to detect and interact with the resistant cells, probably through EGFRWT.

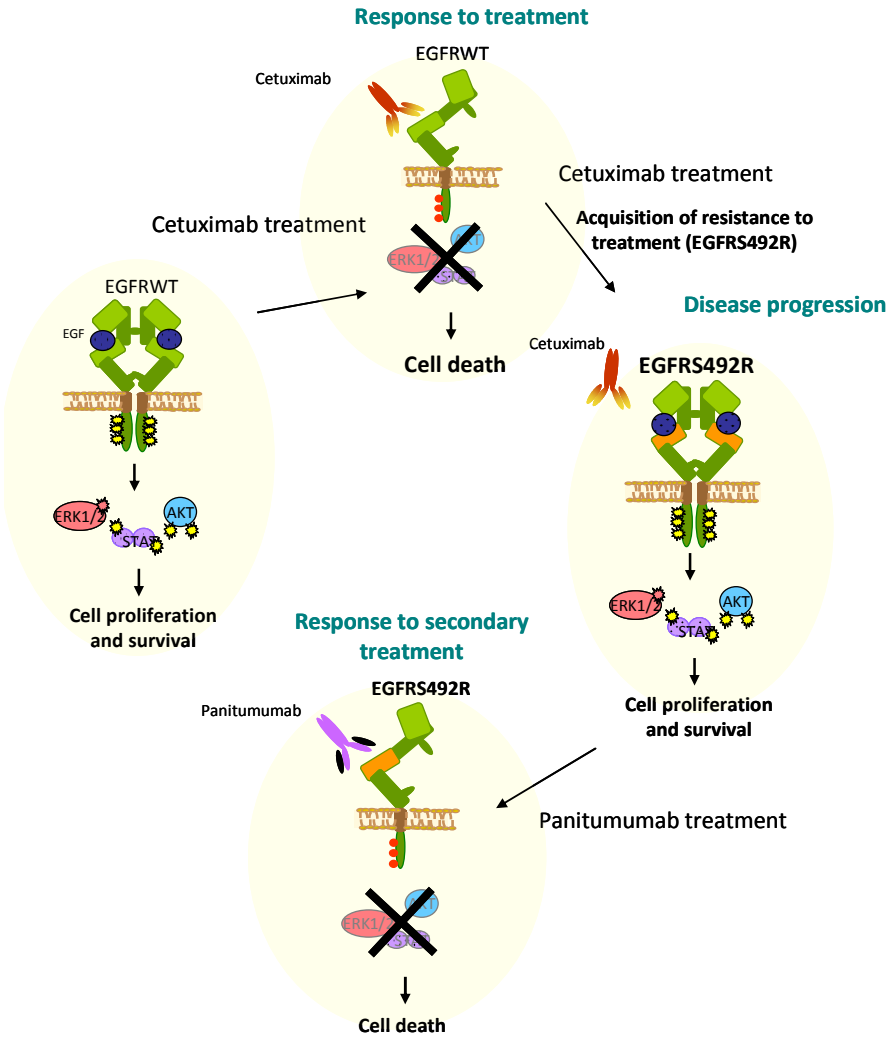


Figure D.1, Schematic representation of the model of acquired resistance to cetuximab. Tumor cells are addicted to EGFR signaling for their survival, when cetuximab is given in a EGFRWT tumor a primary response to the treatment occurs, then, few tumor cells acquire EGFRS492R mutation and tumor progresses. However, if panitumumab is given a secondary response to treatment is observed.

Clinical proof of concept of the results obtained in the preclinical model

To determine whether the results we observed in cell lines were relevant at a clinical level, we analyzed the presence of EGFRS492R mutation in paired tumor samples from patients that initially responded to cetuximab but finally relapsed.

Consistent with our observation in cells, we identified two patients with a tumor that acquired the EGFRS492R mutation following cetuximab treatment. Moreover, both patients harbored *EGFR* gene amplification analyzed by FISH. Thus, the mechanism that we observed in our acquired resistance model (**Figure D.1**) had also occurred in patients. Notably, a patient with a tumor with an acquired EGFRS492R mutation responded to panitumumab after cetuximab failure.

The specificity of the identified point mutation is expected to facilitate reliable testing to guide the clinical use of panitumumab after cetuximab failure, and further prospective validation of the S492R EGFR mutation in colorectal cancer appears to be warranted. It will also be of interest to determine whether this mutation can contribute to the acquisition of cetuximab resistance in other tumors where this drug has been approved, such as head and neck tumors.

Acquired resistance to EGFR TK inhibitors

Although development of resistance to cetuximab is frequently observed in the treatment of colorectal cancer patients, molecular mechanisms underlying such resistance have remained largely

unknown. By contrast, acquisition of resistance to EGFR TK inhibitors (gefitinib and erlotinib, both selective EGFR TK inhibitors) in the setting of lung cancer (NSCLC) has been extensively studied.

Two different mechanisms of resistance have been well characterized and documented in NSCLC. One of them is often due to secondary mutations in the kinase domain, typically, the T790M amino acid substitution affecting the so-called "gatekeeper" position within the catalytic domain that disrupts drug efficacy. This mutation is observed in approximately 50% of the EGFR TK inhibitors resistant cases[173]. The other mechanism, which has been observed in 20% of cases, is due to c-met oncogene amplification, or overexpression of HGF, the c-met ligand, resulting in maintenance of PI3K/AKT pathway by c-met/HER3 dimerization bypassing EGFR signaling[162,163].

Those cell lines and tumors harboring the secondary T790M mutation are still dependent on EGFR signaling; therefore other EGFR TK inhibitors not affected by this aminoacid change, as new-generation irreversible TK inhibitors may still be effective to treat these patients. In the other hand, c-met inhibitors would sensitize those tumors in which c-met oncogene is leading the acquisition of resistance[163].

If we take into consideration the lessons learned in NSCLC, the current knowledge on gefitinib resistance has been achieved by comparison of other TK inhibitors used in other tumor types. Specifically, the determination of whether lung cancers that acquire clinical resistance to gefitinib displayed additional mutations in EGFR kinase domain was based on previous observations with imatinib, an inhibitor of the aberrant BCR-ABL kinase, in chronic myelogenous leukemia. Mutations in the ABL kinase domain were found in 50-90% of patients with secondary resistance to the drug[174]. In addition,

imatinib was also used to inhibit KIT receptor in gastrointestinal stromal tumors where analogous mutations in the kinase domain were found[175]. The analysis of EGFR kinase domain in NSCLC patients with acquired resistance to gefitinib identified the presence of T790M mutation. Therefore, analogous mutations in the TK domain of different RTKs impairing the effects of the TK inhibitors might be a common mechanism of acquired resistance to these agents.

Acquired resistance to monoclonal antibodies in various tumor types

Rituximab, trastuzumab and bevacizumab are moAbs not targeting EGFR approved as therapeutic agents. Rituximab targets CD20, an antigen highly expressed in normal B-Cells and also in B-Cell malignancies promoting cell survival and proliferation. Rituximab is approved for treatment of no-Hodgkin B-cell lymphomas[176]. Trastuzumab is an anti-HER2 antibody approved for treatment of HER2 positive breast cancer[177]. The case of bevacizumab is different, as it is monoclonal antibody against VEGF, which is a soluble factor; it acts as an anti-angiogenic agent more than as a specific antibody against tumor cells. Although the effectiveness of rituximab and trastuzumab is uncontested, primary and acquired resistance to these antibodies has also been observed in the clinical setting and different strategies are being evaluated to overcome resistance to these therapies (reviewed in[178-182]). Alternative variants, as in the case of *EGFRvIII* observed in gliomas, have been described to occur in both CD20[183,184]and HER2[184] leading to rituximab and trastuzumab resistance, respectively. Moreover, activation of alternative RTK such

as IGFR has been proposed as a mechanism of trastuzumab resistance[185]. According to that, we can speculate that similar molecular alterations occur in different membrane receptors which might lead to treatment resistance in different tumor types. However, no mutations in the extracellular domain of CD20 or HER2, as we have seen with EGFR, have already been described in the context of acquired resistance.

Our results give rise to the first study identifying a mechanism of resistance to an anti-tumor antibody which results from a mutation that affects antibody-target binding. So, in light of the large number of therapeutic antibodies that have either been approved for clinical use or are in clinical development, the identification of this novel resistance mechanism suggest that analogous mechanisms in other settings might be occurring. Sequence analysis of CD20 and HER2 antibody binding domain in cell lines and tumors with acquired resistance to rituximab and trastuzumab would be interesting to determine whether the mechanism that we have reported here is also occurring in these frequent malignancies. Other targeting agents of the same proteins could be further therapeutic approaches.

Acquired cetuximab resistance by ectodomain mutation

The mutation described in this PhD thesis is a novel mechanism of resistance to targeted drugs. The EGFRS492R mutation has been acquired in cetuximab sensitive cells under the selective pressure of the treatment, and prevents binding of cetuximab to its epitope within the extracellular domain of EGFR. When analyzing the presence of EGFRS492R in paired tumor samples from patients who initially

benefit from cetuximab therapy but finally become refractory, we observed that tumors from two of ten patients had acquired the mutation. To our knowledge, evidence describing a missense mutation of the extracellular domain of the target of a therapeutic antibody has not been previously reported in the context of treatment resistance.

Recently, it has been shown that aberrant activation of HER2 by either *HER2* gene amplification or heregulin overexpression and consequent HER3 dimerization with HER2, can lead to cetuximab resistance in several cetuximab sensitive cell lines and xenograft models from HNSCC, NSCLC and CRC[186]. Moreover, at a clinical level, *HER2* amplification occurred in two mCRC tumors that had acquired cetuximab resistance and heregulin mRNA levels were significantly higher at the moment of cetuximab relapse compared to the level at the beginning of the treatment[186].

Thus, as in NSCLC treated with gefitinib, mCRC that initially respond to cetuximab acquire resistance to the treatment by either mutating the receptor, as we have seen, or by activating an alternative RTK such as HER2. A reasonable consequence is that other EGFR interacting agents should be considered when the tumor has the EGFRS492R mutation and RTK targeted agents when the resistance is cause by alternative activation of another RTK.

Other potential mechanisms of cetuximab acquired resistance

There are a number of studies that have addressed possible mechanisms of acquired cetuximab resistance in preclinical models. The proposed models have not yet been validated in a clinical setting. However, it is important to discuss them to put into context our

findings since in the years to come the interplay between different resistance mechanisms may have clinical implications. The mechanisms that appear more promising are Src activation, nuclear EGFR and EGFR ligands expression.

The role of Src family of kinases in resistance to RTK targeting agents is currently being evaluated with promising results in different tumor types. It has been proposed that Src activation is a key point to activate alternative RTK when a specific receptor is targeted, therefore combined regimen of Src inhibitors plus targeting antibodies would be more effective than targeting specifically each receptor alone. This observation has been shown in the context of breast cancer and trastuzumab acquired resistance, but it could be extrapolated to cetuximab resistance in colorectal cancer. Specifically, different breast cancer cell lines with acquired resistance to trastuzumab appeared to have overactivation of different RTKs. However, all of them had Src overactivation compared to their respective parental sensitive cell lines, indicating that Src could be a convergence of multiple trastuzumab resistance mechanism. When combining trastuzumab with saracatinib, a Src inhibitor, the sensitivity was restored in all the cell lines and also mouse xenografts[187].

The role of Src on cetuximab resistance has been studied at a preclinical level in NSCLC. It was demonstrated that cetuximab resistant NSCLC cell lines overexpressed EGF family ligands leading to nuclear accumulation of EGFR which impaired cetuximab efficacy. The process from which EGFR was endocyted and transported to the nucleus was mediated through Src, and again, the combined treatment with Src inhibitors and cetuximab could overcome the resistance[188]. In this study, apart from the role of Src in cetuximab resistance, two

other alterations were highlighted that could also be relevant for cetuximab efficacy; EGF family of ligands and EGFR nuclear expression.

As said before, in NSCLC cell lines it was observed that cetuximab resistant cells expressed more EGFR ligands than the parental sensitive cells[188]. Moreover, HB-EGF and TGFα increased in head and neck cancer cell lines by chronic exposure to cetuximab as a mechanism of acquired resistance[189]. According to these preclinical observations, in mCRC patients a general increase of EGFR ligands plasma levels, especially TGFα and amphiregulin, occurred after 4 weeks of cetuximab treatment[190]. All this data suggest that the general increase of ligand expression in response to cetuximab may, in some patients, lead to cetuximab resistance.

On the other hand, nuclear EGFR has been found in highly proliferative tissues and it is associated with worse prognosis in several types of cancer, such as breast and ovary cancer[191-193]. One of the nuclear EGFR's main functions is to act as a co-activator of transcription promoting the expression of several cancer promoting genes, as cyclin D1 and c-Myc[191,194]. EGFR does not contain a DNA binding domain, so it associates with other DNA binding transcription factors in promoter regions. The pathway by which EGF ligands induce EGFR nuclear accumulation requires endocytic traffic through Golgi apparatus and endoplasmatic reticulum[195]. Although this pathway is still poorly understood and characterized clinically, it would lead to cetuximab resistance if it occurred in tumors.

Thus, Src activation, EGFR nuclear expression and EGFR ligands modulation are good candidates to be evaluated in the context of cetuximab primary and acquired resistance.

Targeting EGFR S492R with other EGFR targeting agents

The main goal of understanding the mechanism of acquired resistance is to help on further clinical decisions with alternative therapeutics. Although cetuximab therapy has improved life expectancy of mCRC patients, when a tumor acquires resistance to this moAb no other anti-EGFR drugs are considered in the clinic.

During this PhD project, we have seen that DiFi cells are addicted to EGFR signaling for their survival, and exhibit *EGFR* gene copy number amplification. Indeed, although DiFi cetuximab resistant cells harbored EGFRS492R mutation, they still had *EGFR* gene amplification and were dependent on EGFR for growth and survival. While cetuximab was not able to inhibit EGFR in the resistant cells, both gefitinib and panitumumab effectively inhibited EGFR leading to decreased cell survival. In addition, one patient with a tumor that acquired the EGFRS492R mutation following cetuximab treatment demonstrated a striking response to panitumumab after cetuximab failure. The tumor of this patient, as also observed in DiFi resistant clones, harbored *EGFR* gene amplification, suggesting EGFR addiction. Making a parallelism with NSCLC, where tumors addicted to EGFR signaling are treated with gefitinib and acquire T790M mutation as a mechanism of resistance, one can speculate that in those mCRC addicted to EGFR, commonly associated to *EGFR* gene amplification, the treatment with cetuximab results in EGFRS492R mutation to overcome effects. In this scenario, panitumumab and gefitinib could be an alternative therapeutic strategy to consequently treat those tumors.

Currently, cetuximab and panitumumab, but not gefitinib, are the only two EGFR targeting agents that are used in routine clinical practice for the treatment of colorectal cancer. Cetuximab is a chimeric mouse-human immunoglobulin (Ig) G1 moAb, whereas panitumumab is a fully human IgG2 moAb. Clinically, this translates into distinct toxicity profiles for these two agents. Thus, hypersensitivity reactions following cetuximab administration are rarely seen with panitumumab. Regarding efficacy, both antibodies have demonstrated similar clinical activity[111,115]. Therefore, medical oncologists generally consider these antibodies as equivalent therapies, and are not currently inclined to treat patients with panitumumab following cetuximab treatment failure. On the contrary, a recent report from a single institution experience, described a subset of mCRC patients that had failed cetuximab-based therapy and subsequently responded to panitumumab, without an understanding of the underlying mechanisms of cetuximab resistance[196]. Our findings are consistent with such clinical findings and reveal a potential molecular explanation that has significant clinical implications for the treatment of colorectal cancer patients. Further clinical studies are required to confirm our results which undoubtedly suggest that after treatment failure on cetuximab therapy, patient rebiopsies should be tested for the presence of the EGFRS492R extracellular domain mutation as it may be predictive of benefit associated with subsequent panitumumab therapy.

EGFRS492R was undetected as a mechanism of primary resistance

We have described a mutation in EGFR extracellular domain conferring acquired resistance to cetuximab in cell lines and in patients with mCRC. However, when we have analyzed the presence of the mutation in primary mCRC before receiving cetuximab it was not detected. In addition, EGFR when mutated in position S492R does not seem to become more phosphorylated in basal conditions or after ligand stimulation in NIH3T3 compared to EGFRWT. These findings suggest that S492R mutation does not confer intrinsic oncogenic properties to the receptor and only predicted lack of cetuximab binding. However, we should consider that all DCR clones obtained from DiFi cetuximab sensitive cells acquired the mutation. We cannot fully rule out that a tiny proportion of DiFi parental cells harbored the mutation and by clonal selection they could grow despite the presence of cetuximab. The same may have occurred in those patients that acquired the mutation. However, the percentage of cells with the mutations should be very low as we did not detect the mutation by ultrasequencing, which threshold of detection is below 1%, neither in DiFi nor in the pre-cetuximab treated specimens of those tumors that acquired the mutation. Even considering this, we can not rule out the presence of the mutation in low proportion in primary tumors.

Making again a parallelism in NSCLC drug resistance, clonal selection of c-met amplified or T790M EGFR mutated pre-existent cells occurred with gefitinib resistance[163,170]. When T790M mutation was first described to occur in NSCLC, it did not seem that the receptor had an increased activity and was not found in pretreated tumors[197].

However, later studies demonstrated that the mutation was present in some patients before receiving gefitinib, and EGFR double mutants cells, with gefitinib sensitizing mutations plus T790M mutation, exhibited dramatically enhanced ligand-independent EGFR signaling and transforming phenotypes[170,198].

Thus, although our results suggest that EGFRS492R mutation is not present in primary tumors and does not confer more EGFR activity, further, more specific studies should be done to conclude this result.

Learning from other tumor types to improve molecular classification colorectal cancer

Probably, what we have learned from breast and lung cancer can anticipate what can be achieved in other tumor types, such as colorectal cancer.

The molecular profiling of breast tumors led to the classification of different breast cancer subtypes which can be treated with specific targeted therapies[199,200]. Breast tumors that express hormonal receptors are treated with tamoxifen or aromatase inhibitors[201], which respectively inhibit estrogen receptor and estrogen synthesis, and breast tumors overexpressing HER2 are currently treated with anti-HER2 therapy, such as trastuzumab or lapatinib[177,202]. These targeted therapies have dramatically improved life expectancy of cancer patients that are treated based on the presence of the predictive biomarker in their tumors.

Although anti-EGFR monoclonal antibodies are approved for the treatment of different types of solid tumors, the determination of the molecular alteration that makes the tumor sensitive to these agents is

as yet not known. Therefore, nowadays its clinical use is unspecific as a common chemotherapeutic agent and only mCRC patients with resistant tumors based on the presence of *KRAS* mutations are excluded. Consequently, response rates are low and only few patients really benefit from these agents.

A step ahead in the field of predictive factors of drug benefit has been done in NSCLC and led to the approval of the EGFR TK inhibitor gefitinib specifically in patients with activating EGFR mutations. The first randomized phase III clinical trials of cytotoxic therapy with or without gefitinib in NSCLC patients revealed no survival benefit in gefitinib treated patients[203,204]. However, it was observed that few tumors responded to the therapy in an unselected population. Since it was not further approved in unselected patients, a lot of effort was done later on to identify those patients who would benefit from the inhibitor. Different markers were evaluated, such as EGFR and HER2 overexpression by IHC, but showed no relation with gefitinib efficacy[205].

Later on, EGFR TK domain was sequenced in 119 primary NSCLC tumors. Interestingly, different somatic alterations were found in some tumors; a substitution mutation in L858R located in the ATP binding domain and multiple deletions clustered in the region within the kinase domain of EGFR. Sequence analysis of 9 tumors from patients that received gefitinib revealed no mutations in the four tumors that progressed to the therapy. However, all five gefitinib-responsive tumors harbored EGFR kinase domain mutations[93,94]. According with the observed result at a clinical level, it was also observed in NSCLC cell lines that mutant EGFR cells had EGFR more phosphorylated than WT cells leading to a constitutive activation of the prosurvival

downstream signaling pathways AKT and STAT[95]. These results suggested that EGFR mutant tumors were addicted to the receptor for their survival being 50 times more sensitive to gefitinib than EGFR WT cell lines. Further clinical trials selecting patients with these mutations led to the approval of gefitinib only to treat NSCLC patients harboring EGFR mutant tumors[93,94].

The case of cetuximab in mCRC is different. Although only a 10% of benefit in the response rate was observed, in 2004 it was approved for treatment of mCRC patients[17]. So, on the contrary to gefitinib, despite of its low benefit, cetuximab was approved but no markers of susceptibility or resistance were identified at that time.

Four years later, retrospective analysis of patients treated with cetuximab revealed that 40% of mCRC had *KRAS* mutations which lead to cetuximab resistance[112]. The establishment of mutant *KRAS* as a resistance marker improved cetuximab clinical benefit due to the exclusion of patients with primary resistance.

BRAF and *PIK3CA* mutations may account for another 10-20% of patients that are not responding to the therapy but these markers need further evaluation[156,158].

However, apart from identifying markers of resistance, more effort is needed to identify, as in the case of NSCLC, those tumors that will respond to the therapy. It seems that *EGFR* gene copy number may identify the patients that will benefit, but further validation of the parameters of FISH analysis and studies in larger cohorts of patients are required[99].

If we were able to identify why some tumors are really sensitive to cetuximab we would be able to better understand the outcome of the patients and predict the mechanisms of acquisition of resistance.

Clinical implication of the study

A large-scale effort to analyze patients with acquired resistance to cetuximab is clearly warranted. In such studies secondary tumor biopsies after treatment failure should be systematically performed. Analyses of these tumor tissues are necessary to determine the frequency of the different cetuximab acquired resistance mechanism in patients with mCRC and also to discover additional mechanism.

In addition to the appearance of the EGFRS492R mutation, we observed other genetic changes in the post-cetuximab tumor biopsies that could potentially account for acquisition of resistance to anti-EGFR moAbs. *KRAS* and *BRAF* mutations were found in two different patients after cetuximab failure. Interestingly cetuximab response in the patients with *KRAS* acquired mutation was not evident, only resulting in stable disease. This observation suggests that although we did not detect the mutation in the primary tumor, it might be present in few cells of the primary tumor or in the metastatic sites impairing cetuximab effects. It has been reported that discrepancy between primary tumor and paired metastasis may occur in few cases[206]. Whereas acquisition of a *KRAS* mutation as secondary resistance mechanism to cetuximab has been previously reported[207], to the best of our knowledge, acquisition of a *BRAF* mutation has not been previously described in cetuximab-resistant specimens.

Tumor from patient 9 harbored *BRAF* mutation before and after cetuximab treatment and acquired, at low proportion, EGFRS492R mutation. When cetuximab was given to this patient no major responses were observed, the treatment resulted with stable disease that was maintained during 42 weeks. The results observed in this

tumor might be explained by heterogeneity of cells within the tumor. While those tumor cells with *BRAF* mutation were present before and after the treatment probably leading to partial resistance, the majority of tumor cells with EGFR addiction may have died with cetuximab treatment but a few acquired the EGFRS492R mutation.

Although these findings should be further confirmed as potential mechanisms of acquired resistance, they highlight the need to perform tumor biopsies after drug failure to establish the optimal treatment strategy for each individual patient. If *KRAS* or *BRAF* are mutated, as probably those tumors are not anymore dependent on EGFR, alternative therapy as BRAF inhibitors might be effective. However if EGFRS492R is detected, EGFR targeting agents as panitumumab, that is already approved for mCRC, would be indicated. Other RTK inhibitors might be indicated if aberrant expression of other RTK as HER2, HER3 and c-met is detected, always considering that they should be previously approved for treatment of mCRC.

In summary, understanding the mechanism of drug resistance through both *in vitro* models and in human tumor specimens can clearly lead to the development of more effective therapies and new therapeutic combinations. It will be important to personalize these therapeutic approaches for each patient on the basis of the mechanism of resistance of each individual tumor.

CONCLUSIONS

1. DiFi cetuximab sensitive cells acquired resistance to cetuximab after chronic exposure to the therapy. Cetuximab resistant clones were still dependent on EGFR signaling for their growth and survival. However, cetuximab was not able to inhibit EGFR downstream signalling pathways in the resistant cells.

2. Cetuximab resistant cells acquired EGFRS492R mutation under the selective pressure of cetuximab. The observed aminoacid change impaired cetuximab binding to the receptor; therefore cetuximab was not able to prevent ligand induced phosphorylation.

3. Panitumumab was able to bind EGFRS492R and prevented ligand induced phosphorylation of the receptor. Cetuximab resistant cells with EGFRS492R acquired mutation remained sensitive to panitumumab.

4. Two of ten patients with acquired resistance to cetuximab harbored EGFRS492R mutation.

5. A patient with acquired EGFRS492R mutation responded to panitumumab after cetuximab failure.

MATERIAL AND METHODS

MM.1. CELL LINES AND CELL CULTURE

The DiFi colorectal adenocarcinoma cell line was kindly provided by the Center for Molecular Therapeutics collection at the Massachusetts General Hospital Cancer Center and maintained in DMEM/F-12 from Sigma. LoVo, HTC-116, HT-29, SW-620, SW-48, WiDr and DLD-1 colon adenocarcinoma cell lines were purchased at the American Type Culture Collection (ATCC) and were grown in RPMI media 1640 from Invitrogen. NIH 3T3 mouse embryonic fibroblasts were also obtained from ATCC and were maintained in DMEM media (Invitrogen). All media were supplemented with 2 nM L-glutamine, 100 units/ml Penicillin, 100 μg/ml Streptomycin and 10% FBS. All cell lines were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37ºC.

MM.2. DRUGS AND GROWTH FACTORS

Cetuximab (Erbitux, MERCK) and Panitumumab (Vectibix, AMGEN) were obtained from Hospital del Mar's Pharmacy at 5mg/ml and 20mg/ml, respectively. Both compounds were stored a 4ºC. Gefitinib was obtained from Selleck Chemicals (Houston, TX, USA) and was dissolved in DMSO at a final concentration of 10mM, it was aliquoted and stored at -20ºC. Purified EGF and TGFα recombinant proteins were purchased from Calbiochem (San Diego, CA, USA). They here dissolved in PBS 0,1% BSA at 100μg/mL, aliquoted and stored at -80ºC.

MM.3. GENERATION OF CETUXIMAB-RESISTANT COLON CANCER CELLS

DiFi cells were chosen to generate cetuximab resistant cells. Approximately $1x10^5$ DiFi cells were seeded in each of three 10-cm dishes. Fresh culture media containing 1 μg/mL cetuximab was added to the cells every third day. Four months after drug selection, approximately 10 clones per dish appeared. Single cell clones were isolated using cloning cylinders of 8mm diameter (Millipore) and were further propagated for characterization in media containing 1 μg/mL cetuximab.

MM.4. CELL PROLIFERATION AND VIABILITY

To asses the effects of different drugs in cell lines we used different approaches; MTS assay, which measures the metabolic activity of cells, manual cell counting and crystal violet staining to determine the number of living cells and cell cycle distribution by flow cytometry, to determine the percentage of cells in each cell cycle phase.

MM.4.1. MTS assay

Cell proliferation and viability was studied using the colorimetric method provided by the MTS-CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega). MTS assay is a modification from MTT assay and depends on two solutions: a tetrazolium compound (MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan

product. Cells that are metabolically active can accomplish by dehydrogenase enzymes the conversion of MTS into aqueous, soluble formazan product. The quantity of formazan product measured by the amount of 490nm absorbance is directly proportional to the number of living cells in the culture. We performed this viability assay in 96-well flat-bottomed plates. Approximately $1x10^4$ cells were seeded in 100μl of culture media in each well and incubated during 24h before drug treatment. Next, 100μl of 2x drug containing media was added in each well. After 48hours, 20μL of MTS/PMS reagent was added to each well and the cells were further incubated from 1 to 4 hours more. The amount of soluble formazan was measured on a microplate spectrophotometer at 490nm (test wavelength) and 690nm (reference wavelength). The percentage of surviving cells to each treatment was estimated by dividing the A490nm- A690nm of treated cells with the A490nm- A690nm of control cells.

MM.4.2 Manual cell counting

Cell proliferation and viability was alternatively studied by manual counting of the cells. Approximately $1,7x10^6$ cells were seeded in 10mm culture plates and after 24 hours were treated with cetuximab or panitumumab 10μg/ml. After 48h of treatment cells were harvested with PBS and tripsinized. The number of viable cells in each condition was determined by counting the cells stained with trypan blue die solution in a hemocytometer chamber. The number of viable cells in each treatment was related to the number of viable cells in the control condition.

MM.4.3.Crystal violet staining of cells

For Crystal Violet staining, approximately $3x10^5$ cells were seeded in MW6 plates. After 24 hours, cells were treated with cetuximab or panitumumab 10μg/ml for 72 hours. Culture media was carefully removed and the wells were washed with PBS. Then, Crystal violet solution (10% Acetic acid, 10% Absolute Ethanol and 0,06% Crystal violet) was added in an amount sufficient to cover the surface culture area during at least 1hour. After that, the plates were washed five times by filling the wells with PBS. Plates were inverted and dried on paper towels. Images of each plate were scanned and analyzed by ImageJ software. The percentage of growth was calculated by dividing the mean value of treated cells by the mean value of untreated cells.

MM.4.4. Cell cycle analysis by Flow cytometry

For cell cycle distribution analysis, approximately $1,7x10^6$ cells were seeded in 10mm plates and 24hours later cells were treated 24 more hours with cetuximab 10μg/ml. Cells were harvested by tripsinitzation and counted. After washing three times with PBS by 8 minutes of centrifugation at 1500 rpm's, approximately $1x10^6$ cells were resuspended and fixed on 70% ethanol with PBS overnight. Ethanol was removed by washing 3 times with PBS, by 10.000rpms 2 minutes, and DNA was stained with PBS containing 50μg/ml propidium iodide and 100μg/ml of RNAse at least during 48h at 4ºC protected from light. Cell cycle distribution was analyzed using a FACScalibur flow cytometer (Beckton Dickinson) with Cell Quest Software.

Sample running, data acquisition and interpretation was performed under the supervision of the flow cytometry core facility staff.

MM.5. PROTEIN DETECTION

MM.5.1. Protein extraction

MM.5.1.2. Total protein extraction

For whole cell lysates, cells were cultured in plates 10mm plates and after treatment at each specific condition, plates were washed with cold PBS to remove traces of remaining culture media. Then, cells were lysed with 100µl of ice-cold Nonidet P-40 buffer (Tris-HCL (pH = 7.4) 50mM, NaCl 150mM, 1 % NP40, EDTA 5mM, NaF 5mM, Na3VO4 2mM, PMSF 1mM, Leupeptin 5µg/mL and Aprotinin 5µg/mL) mechanically with the help of an scrapper. Cell lisate was transferred to an eppendorf. After shaking during 30min at 4ºC, the samples were centrifuged 30min at 13200rpm and the supernatant was aliquoted and stored at -20ºC.

MM.5.1.2 Nuclear and cytoplasmic proteins extraction

For nuclear an cytoplasmic extracts, cells were cultured in 150mm plates, then were washed in PBS and removed from the dish with the scrapper. After centrifugation pellet was resuspended and lysed in Buffer A (20mM Hepes pH8 10mM KCl 0'15mM EDTA pH 8, 0'15 mM Espermidina, 0'15 mM Espermina , DTT 1mM, 1,2% Triton and protease inhibitors) using a 25-G syringe. Then cytoplasmic fraccion was excluded from the nucleus, sucrose restore buffer was added

(50mM Hepes ph7, 0.25mM EDTA pH8, 10mM KCl and 70% Sucrose) and samples were centrifuged 5 minutes at 5000rpm. Supenatant contained the cytoplasmic fraction, while pellet was the nuclear extract.

- Supernatant was centrifuged 15 minutes at 14000rpms and the supernatant fraction, was aliquoted, 15% of glycerol was added and stored at -80ºC (cytoplasmic extract).
- Pellets were resuspended in Buffer B (20mM Hepes pH8, 50mM NaCl, 25% Glicerol, 0.15mM EDTA pH8, 0.15mM EGTA pH8, 1.5mM $MgCl_2$) and centrifuged again 15min more. Pellet was resuspended in buffer B plus 400mM of KCL and then samples were shaked on ice during 30minutes. Finally all samples were centrifuged at 10000rpm 5 minutes and supernatant was collected and stored at -20ºC.

The quantification of total protein in the cell lysates was done with Bio-rad DC protein assay (Bio-Rad) which is based in Lowry assay.

MM.5.2. Western Blot analysis

To determine the expression of specific proteins in different cells or different conditions, the same quantity of protein for each sample, usually 30μg, was adjusted to the same volume with lysis buffer. An equal amount of 2x 5% β-mercaptoethanol Laemmly-sample buffer was added to each sample. Samples were denaturalized by 5 minutes at 95ºC.

Samples (30μg/lane) were subjected to SDS-page and transferred to PDVF membranes. Immunoblotting was carried out according to standard procedure. To block unspecific detection, PDVF membranes

were incubated with 5% Milk TBS-T for 1 hour at room temperature. Then, membranes were incubated overnight at 4ºC with the specific primary antibody (listed below). After washing 3 times with TBS-T, horseradish peroxidise-conjugated secondary antibodies from Amersham were used to detect primary antibodies. Again, membranes were washed 3 times with TBS-T and target proteins were visualized after enhanced chemiluminescence treatment (Amersham) of membranes and subsequent exposure to X-ray film. The following primary antibodies were used for protein detection: phospho EGFR (Tyr1068), phospho ERK1/2 (Thr202/Tyr204), phospho AKT (Ser473), EGFR, ERK1/2 and AKT were purchased from Cell Signalling Technology. Anti-α-Tubulin was from Sigma. Cyclin D1 was from NeoMarkers. PARP-1 was kindly provided by Jose Yélamos.

MM.5.3. Phospho Receptor tyrosine-kinase Array

We screened the activity of a panel of different Tirosine Kinase Receptors using an antibody based array, phosphoRTK array from R&D Systems. Antibodies against 42 different RTKs were prespotted on nitrocellulose membranes. 50 μg of cell lysates from parental DiFi and DCRs were incubated with the membranes overnight. After washing 3 times with TBS-T, a pan antiphosphotyrosine antibody was used to detect the phosphorylated tyrosine residues of the RTKs. Membranes were visualized after chemiluminescence treatment and subsequent exposure to X-ray film.

MM.6. EGFR mRNA SEQUECING

Total RNA from DiFi and DCRs was isolated using RNeasy mini Kit (Qiagen, GMBH, Hilden, Germany) following manufacturers instructions. The amount of RNA per sample was quantified using the nanodrop. After extraction the RNA was reverse transcribed to cDNA using HighCapacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following manufacturers instructions. The primers described in **Table MM1** were designed using Primer Express software (Applied Biosystems) using NM_005228.3 sequence to amplify with Ampli TAQ GOLD (Applied Biosystems) EGFR cDNA specific fragments of around 100bp covering all CDNA sequence.

Table MM1. Primers sequence of primer designed to amplify all EGFR cds.

EGFR cDNA	Primer Forward	Primer Reverse
Fragment 1	5'-TCCGTCCAGTATTGATCGGG	5'-GGATCACACTTTTGGCAGCTG
Fragment 2	5'-TGCCCATGAGAAATTTACAGGA	5'-CATCTCATAGCTGTCGGCCC
Fragment 3	5'-GCCCCCCACTCATGCTCTA	5'-GGATGTTATGTTCAGGCTGACGA
Fragment 4	5'-CCTAGAAATCATACGCGGCAG	5'-GGGTGTTGTTTTCTCCCATGAC
Fragment 5	5'-TGCCCACTACATTGACGGC	5'-TCACGTAGGCTTCATCGAGGAT
Fragment 6	5'-GGGCTCCGGTGCGTTC	5'-CCCCGTAGCTCCAGACATCA
Fragment 7	5'-CACCGCGACCTGGCA	5'-ATGAGGTACTCGTCGGCATCC
Fragment 8	5'-TCCGTGAGTTGATCATCGAATT	5'-GGGCTGGACAGTGTTGAGATACT
Fragment 9	5'-ACACCTTCCTCCCAGTGCCT	5'-TGTGCCCGAGGTGGAAGTAC

DNA amplification was performed by PCR under the following conditions:

1- Initial denaturation for 10 min at 95ºC
2- 40 cycles consisting of:
    a. 1 min at 95ºC
    b. 1 min at 60ºC

c. 1 min at 72ºC

3- A final step at 72ºC for 10 min.

Then, each PCR product was purified with vacuum columns and sequenced using BigDye v3.1 (Applied Biosystems) following the manufacturer's instructions. Sequences were analyzed on an ABI3730XLSequencer (Applied Biosystems).

MM.7. EXPRESSION CONSTRUCTS, TRANSFECTION AND INFECTION

The vectors pBABE-puro empty and pBABE-puro cloned with EGFR cDNA were obtained from Addgene. 1476C_A point mutation was generated using the Quick-Change Site Directed Mutagenesis kit (Agilent Technologies) with the following oligonucleotide primers:

Fw 5'-CGGTCAGAAAACCAAAATTATAAGAAACAGAGGTGAAAACAGCT

Rv 5'-AGCTGTTTTCACCTCTGTTTCTTATAATTTTGGTTTTCTGACCG

Retroviral infection was used to generate NIH3T3 cells with stable ectopic expression of WT and mutant EGFR.

- Day 0: $1x10^6$ 293T-derived Phoenix packaging cell lines were seeded in 6mm culture plates.
- Day 1: EGFR pBABE-puro constructs (Empty, EGFRWT, EGFRS492R) were transfected into 293T-derived Phoenix packaging cells using lipofectAMINE 2000 (Invitrogen), following manufacturers instructions. After 6 hours, medium was removed and fresh media was added.
- Day 2: After 24 hours of transfection media from 293T-derived Phoenix packaging cells was remove and fresh media was added to

accumulate retroviral supernatant. In parallel, $1x10^5$ NIH3T3 cells were seeded in 6mm culture plates.

- Day 3: 293T-derived Phoenix packaging cell derived media was centrifuged at 1500 rpm's for 5 minutes. Viral supernatant was filtered with 0,45 μm filter and 1 ml was added to NIH3T3 cells in the presence of polybrene.
- Day 4: NIH3T3 cells media was removed and fresh media was added.
- Day 5: Two days after infection puromycin 2μg/ml was added and surviving cells were selected to derive pooled stable cell lines.

MM.8. CETUXIMAB AND PANITUMUMAB BINDING TO EGFR

The ability of different anti-EGFR monoclonals antibodies to bind EGFRWT and EGFRS492R was determined by three different approaches; Flow cytometry, Immunoprecipitation and ELISA.

MM.8.1. Cetuximab and panitumumab binding by Flow Cytometry

To measure cetuximab and panitumumab binding to EGFR cells were harvested by tripsinization and washed twice with PBS by centrifugation at 1500rpm's 8minutes. Fc blocking solution was incubated to the cells for 15 minutes on ice to block unspecific Fc binding of Immonuglobulins. Cells were washed again with PBS and incubated with 100ng/mL of the monoclonals antibodies, cetuximab, panitumumab and irrelevant IgG, for EGFR binding for 30 minutes

on ice. To visualize the primary antibody a goat anti-human IgGγ conjugated with Phycoeritrin (Invitrogen) was used as a secondary antibody. EGFR binding was analyzed using the FACScan Flow Cytometer (Beckton Dickinson).
Sample running, data acquisition and interpretation was performed under the supervision of the flow cytometry core facility staff.

MM.8.2. Immunoprecipitation

Cell were seeded in 150mm culture plates and after 24 hours were washed twice with ice cold PBS and lysed with coimmunoprecipitation buffer (20mM sodium phosphate pH7.4, 150mM NaCl, 1% NP40, 10% Glycerol, 1mM EDTA, 5mM phenylmethylsulfonyl fluoride, 10μg/mL aprotinin and leupeptin, 250μg/ml sodium orthovanadate and 40mM NaF). Then, cells were centrifuged during 10 minutes at 14000 rpms and 200μl of supernatant fraction was incubated overninght at 4ºC with 10μg/mL of cetuximab or panitumumab or unspecific IgG. After primary antibody binding, lysates were incubated 3 more hours at 4ºC with 20μL of ProteinA/ProteinG agarose beads (Calbiochem). The immuncomplexes were pelleted by centrifugation, washed five times with coimmunoprecipitation lysis buffer and resuspended with 20 μl of Laemli sample buffer. Samples were boiled 5min at 95ºC and were subjected to SDS-Page electrophoresis and Western Blot analysis.

MM.8.3. In vitro antibody binding assays

Competitive binding assay: Anti-EGFR Ab binding to wild-type (WT) and mutant extracellular domain (ECD) was compared in a competitive sandwich ELISA. Recombinant EGFR ECD human Fc fusion protein (WT or mutant) was immobilized onto a plastic surface overnight. The plate was then blocked with PBS containing BSA. Anti-EGFR Abs and a negative control huIgG Ab were serially diluted and mixed with an equal volume of biotin-labeled Panitumumab at fixed concentration; the mixture was added onto the plate. The sample was incubated for 2 hours. The plate was washed and streptavidin-horseradish peroxidase (SA-HRP) conjugate was added as detection. The substrate tetramethyl benzidine (TMB) was added to the plate, and the reaction was stopped with acid. The plate was read and the Ab competitive binding results from WT and mutant ECD were compared.

Direct binding assay: Anti-EGFR Ab binding to WT and mutant ECD was compared in a sandwich ELISA. Recombinant EGFR ECD human Fc fusion protein (WT or mutant) was immobilized onto a plastic surface overnight. The plate was then blocked with PBS containing BSA. Anti-EGFR Abs were diluted and added onto the plate to allow binding to occur for 2hours. The plate was washed and a goat anti-human IgG, $F(ab')_2$ fragment specific-Horseradish Peroixdase (HRP) conjugate was used as detection in the assay. The colorimetric substrate tetramethyl benzidine (TMB) was added to the plate, and the reaction was stopped with acid. The plate was read and the Ab binding results from WT and mutant ECD were compared.

MM.9. TUMOR SAMPLES AND PATIENTS

Eighty two tumor specimens obtained during diagnose or surgical procedures on metastatic colorectal cancer patients subsequently treated with cetuximab-based therapy between the years 2004 and 2010 at Hospital del Mar were retrospectively collected from Parc de Salut MAR Biobank (MARBiobanc), Barcelona. Patient selection criteria were based on tumor tissue availability for molecular analysis. In addition, CT guided biopsy of tumor lesions from patients that demonstrated tumor re-growth (disease progression) after initial response to cetuximab-based therapy were collected whenever possible at the time of progression. Biopsy was obtained from the most accessible malignant lesion (either primary tumor or metastasis) with less potential risk of related complications. This study was approved by the local Ethics Board (CEIC-IMAS2009/3515/I). Informed consent was obtained from all patients. DNA extraction and sequencing was performed as described above.

MM.10. MUTATIONAL STATUS OF DIFFERENT MARKERS

MM.10.1. Direct sequencing of *KRAS, BRAF, PI3KCA* and *EGFR*

DNA was extracted from two 15μm sections of paraffin-embedded tissue using the QIAamp Tissue Kit (QIAGEN) according to the manufacturer's protocol. In those cases in which < 50% of tumor cells were present in the sample, manual microdissection of tumor tissue

was performed. Primers for *KRAS* (codons 12 and 13), *BRAF* (V600E), *PIK3CA* (exon 9 and 20) and *EGFR* (S492R) were designed using Primer Express software (Applied Biosystems) using NG_007524.1 (*KRAS*), NG_007873.1 (*BRAF*), NG_012113.1 (*PI3KCA*) and NG_007726.1 (*EGFR*) sequences and were as follows:

KRAS Fw: 5'-TTACGATACACGTCTGCAGTCAAC-3'
KRAS Rv: 5'-AAAGAATGGTCCTGCACCAGTAATA-3'

BRAF Fw: 5'-CGGCTCCTAAAGCAATGGC-3'
BRAF Rv: 5'- CAGCATCTCAGGGCCAAAAA-3'

PIK3CA Ex 9 Fw: 5'-TTATGTCTTAGATTGGTTCTTTCCTGTC-3'
PI3KCA Ex9 Rv: 5'-ATGGAGATTCTCTGTTTCTTTTTCTTTATT-3'

PI3KCA Ex 20 Fw: 5'-GCCTCTCTAATTTTGTGACA-3'
PI3KCA Rv 20 Rv: 5'-AAATGAAAGCTCACTCTGGATTCC-3'

EGFR Fw: 5'-CAAAGTTTTCAGGGATACATTGTTTTT-3'
EGFR Rv: 5'-CGGTGACTTACTGCAGCTGTTTT-3'

DNA amplification was performed by PCR under the following conditions:

1- Initial denaturation for 10 min at 95ºC
2- 40 cycles consisting of:
    a. 1 min at 95ºC
    b. 1 min at 54ºC (KRAS) ,at 55ºC (BRAF), 52ºC(PI3KCA Ex9) at 55ºC (PI3KCA Ex20) and at 60ºC (EGFR)
    c. 1 min at 72ºC
3- A final step at 72ºC for 10 min.

Mutational analysis was performed by direct sequencing with BigDye v3.1. (Applied Biosystems) according to the manufacturer's instructions and analyzed on an ABI3730XLSequencer (Applied Biosystems).

MM.10.2 EGFR S492R determination by qPCR

To detect EGFRS492R mutation by quantitative PCR we designed three different TaqMan probes conjugated with different dyes, VIC and FAM, from Applied Biosystems:

- EGFR WT: CACCTCTGTTGCTTATAA- VIC
- EGFR 1476C>A: CACCTCTGTTTCTTATAATT- FAM
- EGFR 1474A>C: CTCTGTTGCGTATAATT- FAM.

DNA amplification was done with EGFR primers (described in MM.10.2) in a 7500 Real Time PCR-System (Applied Bioscience) using TagMan Universal Master MIX II (Applied Bioscience) and the TaqMan probes under the following conditions:

1- Initial step for 2 minutes at 50ºC.
2- A denaturation step for 10 min at 95ºC
3- 40 cycles consisting of:
    a. 15 seconds at 95ºC
    b. 1min at 60ºC

The WT probe together with the mutated (1476C>A or 1474 A>C) were used within the same reaction to see their relative presence in the same sample.

MM.10.3. Sequencing library preparation and Illumina sequencing

We designed the specific following pairs of primers to generate different PCR products, all covering EGFR S492R sequence, in order to distinguishing the resulting DNA product from each sample:

Fw1: 5'-CTCCGGTCAGAAAACCAAAATT-3'
Rv1: 5'-GGACCCATTAGAACCAACTCCA-3'

Fw2: 5'AAAAACTGTTTGGGACCTCCG-3'
Rv2: 5'-ACTCCATAAACTAAACAGAAAGCGG-3'

Fw3: 5'-ACTGTTTGGGACCTCCGGTC-3'
Rv3: 5'-ACCAACTCCATAAACTAAACAGAAAGC-3'

Fw4: 5'-ACCTCCGGTCAGAAAACCAA-3'
Rv4: 5'-ACCCATTAGAACCAACTCCATAAACTA-3'

Fw5: 5'-GACCTCCGGTCAGAAAACCA-3'
Rv5: 5'-CCCATTAGAACCAACTCCATAAACTAAA-3'

Fw6: 5'-TGGAAAAAACTGTTTGGGACCT-3'
Rv6: 5'-CATAAACTAAACAGAAAGCGGTGACTT-3'

Fw7: 5'-GTTTGGGACCTCCGGTCAG-3'
Rv7: 5'-AGAACCAACTCCATAAACTAAACAGAAA-3'

Fw8: 5'- GGACCTCCGGTCAGAAAACC-3'
Rv8: 5'-CCATTAGAACCAACTCCATAAACTAAACA-3'

Fw9: 5'-ATACAATAAACTGGAAAAAACTGTTTGG-3'
Rv9: 5'-CAGAAAGCGGTGACTTACTGCA-3'

Fw10: 5'-TAAACTGGAAAAAACTGTTTGGGAC-3'
Rv10: 5'-ACTAAACAGAAAGCGGTGACTTACTG-3'

Fw11: 5'-TTGGGACCTCCGGTCAGA-3'
Rv11: 5'-TTAGAACCAACTCCATAAACTAAACAGAA-3'

Fw12: 5'-GGGACCTCCGGTCAGAAAA-3'
Rv12: 5'-CATTAGAACCAACTCCATAAACTAAACAGA-3'

Fw13: 5'-AACTGTTTGGGACCTCCGGT-3'
Rv13: 5'-ACCAACTCCATAAACTAAACAGAAAGC-3'

Fw 14: 5'- ACTGGAAAAAACTGTTTGGGACC-3'
Rv 14: 5'-CATAAACTAAACAGAAAGCGGTGACTTAC-3'

DNA amplification was performed by PCR under the following conditions:

1- Initial denaturation for 2 min at 95ºC
2- 45 cycles consisting of:
    a. 45 seconds at 95ºC

b. 45 seconds at 56ºC

c. 1min at 72ºC

3- A final step at 72ºC for 10 min.

Sequencing libraries were prepared by ligation of Illumina adapters to PCR fragments according to published procedures[208]. Briefly, the ends of DNA fragments were made blunt by end-repair with T4 DNA polymerase and Klenow fragment. Thereafter, 3'-adenylation was performed by incubation with dATP and exo- Klenow fragment. Double-stranded adapters were ligated to the DNA using rapid T4 DNA ligase. On a 2% agarose gel, fragments in the size range of interest were excised with a sterile scalpel and recovered from the gel. Adapter-ligated fragments were enriched, and adapters were extended with an 18-cycle PCR reaction. Finally, the library quality was confirmed on the Agilent 2100 Bioanalyzer, and loaded onto a v5 single read flowcell (IIllumina) at a concentration of 7-8pM. Clusters were prepared on the Illumina cluster station. The flowcell was loaded onto the Illumina Genome Analyzer IIx, and 36 sequencing cycles were performed using Illumina TrueSeq sequencing chemistry. Basecalling was performed using Illumina RTA version 1.7.0 (within SCS2.9). Two lanes on two different flowcells were run from amplicon pools.

Reads were selected that contained a perfect match to either the forward or to the reverse PCR primer that had been used to prepare amplicons from the EGFR gene. Seventy-five percent of 89 million raw sequences could be assigned to primer sequences, tolerating zero mismatches. Thereafter, read filtering was performed, i.e. chastity filtering, removal of reads with uncalled bases and removal of reads with low quality bases. 7-13% of the reads that had a perfect match to

one of the PCR primers were removed in this filtering step. The final data set for each EGFR primer, after read filtering, contained between 0.3 and 2.9 million reads (average 1.2 million reads).

For each primer, reads were clustered and counted using a custom perl script, in order to determine the most frequent base changes in comparison to the EGFR reference sequence. PCR co-amplification products affecting four out of 16 amplicons were identified by the high number of mismatches (>=4 mismatches per read), and were excluded from further analysis. As an additional quality threshold, sequence clusters were ignored if the base differing from the reference sequence showed a decreased average quality value. The average quality value Q assigned by Illumina for bases of reads passing all the filtering steps was Q=38. Among the three biggest sequence clusters associated to each of the 32 primer sequences, nine showed a decreased average quality score of <20 at the mismatched base. These base changes are potentially sequencing errors and cannot be trusted. One of the 16 AGC triplets that were surveyed for substitutions contained such a base change with decreased quality. The corresponding read cluster was discarded.

To find a cutoff to distinguish between sequence variants and sequencing errors, we made use of the sequences assigned to the primers opposite to the one at which we expect a potential mutation in the AGC triplet. For 13 of these 16 primers, the largest sequence cluster not corresponding to the reference sequence represented 0.1-0.6% of the assigned reads. For the three remaining primers the largest non reference sequence cluster were 81.6%, 67.2% and 3.4%. The latter three represent true sequence variants, present in a subset of the sample. Based on this we define a mutation to be detectable if it is

present in more than 1% of the reads, at an average quality value of Q>35 at the mismatched position. Base changes present in less than 1% of the reads cannot be distinguished from random sequencing errors, even when applying high thresholds for base quality values.

MM.11. FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

To assess the genetic status of the *EGFR* gene in both cell lines, we applied the FISH technique by using the LSI EGFR/CEP7 commercial probe (Abbott Molecular Inc). This probe consists of two different probes, one with the centromeric alpha-satellite region, specific for chromosome 7 (Spectrum green), and a locus specific probe from the 7p12 region, that contains the *EGFR* gene (Spectrum orange). FISH was performed in Carnoy fixed cells (suspension of nuclei and metaphases) obtained from the cell lines after application of a conventional cytogenetic protocol. Samples and the probe were co-denaturated at 75ºC for one minute and hybridized overnight at 37ºC in a hot plate (Hybrite chamber, Abbot Molecular Inc.). Post-hybridization washes were performed at 45ºC in a series of 50% formamide, 2xSSC and 2xSSC 0.1% NP-40 solutions for 10 minutes. Samples were counterstained with 4,6-diamino-2-phenilindole (DAPI) (Vysis, Inc.). Results were analyzed in a fluorescent microscope (Olympus, BX51) using the Cytovision software (Applied Imaging). A minimum of 200 non-overlapping nuclei per case was analyzed. The parameters of FISH scoring were according to the following parameters described in[169] (**Table MM2**).

Table M.M.2. Parameters of FISH scoring

Category	*Cappuzzo et al, 2005*	FISH status
1	**Disomy:** 2 or less than two copies of EGFR gene in 90% or more cells	FISH negative
2	**Low trisomy:** 2 or less than two copies of EGFR gene in 40% or more cells 3 copies of EGFR gene in 10 to 40% of cells 4 or more copies of EGFR gene in less than 10% of cells	
3	**Low trisomy:** 2 or less than two copies of EGFR gene in 40% or more cells 3 copies of EGFR gene in 40% ore more cells 4 or more copies of EGFR gene in less than 10% of cells	
4	**Low polisomy**: More than 4 copies of EGFR gene in 10 to 40% of cells	
5	**High polisomy**: More than 4 copies of EGFR gene in 40% or more of cells	FISH positive
6	**Amplification:** EGFR Clusters or Ratio EGFR/CEP7 from 2 or more or $\geq$15 copies of EGFR in 10% or more cells	

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PUBLICATIONS

PUBLICATIONS PRESENTED IN THIS PhD THESIS:

This PhD Thesis has given rise to the following publication:

Authors: Montagut C*, **Dalmases A***, Bellosillo B, Crespo M, Pairet S, Iglesias M, Salido M, Gallen M, Marsters S, Tsai SP, Minoche A, Somasekar S, Serrano S, Himmelbauer H, Bellmunt J, Rovira A, Settleman J*, Bosch F* & Albanell J*.

* Equal contribution

Title: *Identification of a mutation in the extracellular domain of Epidermal Growth Factor Receptor mutation conferring cetuximab resistance in colorectal cancer.*

Journal: Nat Med 2012 [doi:10.1038/nm.2609]

PUBLICATIONS NOT PRESENTED IN THE PhD THESIS:

The following publications have been done during the period of the PhD Thesis as a collaborator:

Authors: Inglés-Esteve J, Morales M, **Dalmases A**, García-Carbonell R, Jené-Sanz A, López-Bigas N, Iglesias M, Ruiz-Herguido C, Rovira A, Rojo R, Albanell J, Gomis R, Bigas A, Espinosa L.

Title: *Inhibition of specific NF-kappaB activity contributes to the tumor suppressor function of 14-3-3sigma in breast cancer.*

Journal: Under revision

Authors: Montagut C, Iglesias M, Arumí M, Bellosillo B, Gallén M, Martínez-Fernández A, Martínez-Avilés L, Cañadas I, **Dalmases A**, Moragon E, Lema L, Serrano S, Rovira A, Rojo F, Bellmunt J, Albanell J.

Title: *Mitogen-activated protein kinase phosphatase-1 (MKP-1) impairs the response to anti-epidermal growth factor receptor (EGFR) antibody cetuximab in metastatic colorrectal cancer patients.*

Journal: Br J cancer 2010; 102: 7 1137-1144

Authors: Rojo F, González I, Bragado R**, Dalmases A**, Menéndez S, Cortes-Sempere M, Suárez C, Oliva C, Rodriguez-Fanjul V, Sáncehz-Pérez I, Corominas JM, Tusquets I, Bellosillo B, Serrano S, Perona R, Rovira A, Albanell J.

Title: *Mitogen-activated protein kinase phosphatase-1 (MKP-1) in human breast cancer independently predicts prognosis and is repressed by doxorubicin.*

Journal: Clin Cancer Res 2009: 10; 3530-3539

Authors: Rojo F, **Dalmases A**, Corominas JM, Albanell J.

Title*: Pharmacodynamics: biological activity of targeted therapies in clinical trials.*

Journal: Clin Transl Oncol 2007: 9; 634-644

Authors: Tapia MA, González-Navarrete I, **Dalmases A**, Bosch M, Rodriguez-Fanjul V, Rolfe M, Ross JS, Mezquita J, Mezquita C, Bachs O, Gascón P, Rojo F, Perona R, Rovira A, Albanell J.

Title: *Inhibition of the canonical IKK/NF Kappa B pathway sensitizes human cancer cells to doxorubicin.*

Journal: Cell Cycle 2007: 18; 2284-2292

Authors: Albanell J, **Dalmases A**, Rovira A, Rojo F.

Title*: mTOR signalling and cancer.*

Journal: Clin Transl Oncol 2007: 8; 484-493

Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, Salido M, Gallen M, Marsters S, Tsai SP, Minoche A, Somasekar S, Serrano S, Himmelbauer H, Bellmunt J, Rovira A, Settleman J, Bosch F, Albanell J. **Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer.** Nat Med. 2012 Jan 22; 18(2):221-3.

Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, Salido M, Gallen M, Marsters S, Tsai SP, Minoche A, Somasekar S, Serrano S, Himmelbauer H, Bellmunt J, Rovira A, Settleman J, Bosch F, Albanell J. **Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. Supplementary information.** Nat Med. 2012 Jan 22; 18(2):221-3.