







**Table 1.** EGFR pathway mutations in tissue samples from patients with mCRC treated with cetuximab

Patient #	Pre-treatment										Post-treatment															
	KRAS exon 2	KRAS exon 3	KRAS exon 4	NRAS exon 2	NRAS exon 3	NRAS exon 4	BRAF exon 15	PIK3CA exon 9	PIK3CA exon 20	EGFR exon 12	KRAS exon 2	KRAS exon 3	KRAS exon 4	NRAS exon 2	NRAS exon 3	NRAS exon 4	BRAF exon 15	PIK3CA exon 9	PIK3CA exon 20	EGFR exon 12						
																					R451C	S464L	G465R	K467T	I491M	S492R
4																										
5																										
8																										
11																										
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NOTE: KRAS, NRAS, BRAF, PIK3CA, and EGFR mutations were analyzed in paired tissue samples obtained at diagnosis (pre-treatment) and at progression (post-treatment). Black boxes indicate the presence of mutations detected by clinical routine sequencing procedures.

mutations). Of note, mutations in *PIK3CA* always coexisted with other mutations within the same sample.

Notably, in addition to "RAS" gene mutations the molecular analysis revealed the emergence of multiple alleles in the EGFR extracellular domain. The previously reported *EGFR S492R* variant was detected in 3 patients. Of note, *EGFR* mutations were accompanied by gene amplification in both the pre- and the posttreatment specimen from two of these patients (data not shown); in the third patient, *EGFR* gene copy number was not assessed due to lack of sufficient tissue for FISH analysis.

Two novel mutations located in exon 12 of the *EGFR* gene were discovered by the analysis of posttreatment samples. Patient #31 harbored an A → C substitution at codon 1400 that caused a substitution of a lysine to threonine at amino acid 467 (p.K467T). The postcetuximab biopsy from patient #35 harbored a C → T substitution at codon 1351, resulting in an arginine to cysteine substitution at amino acid 451 (p.R451C; Table 1; Supplementary Fig. S1).

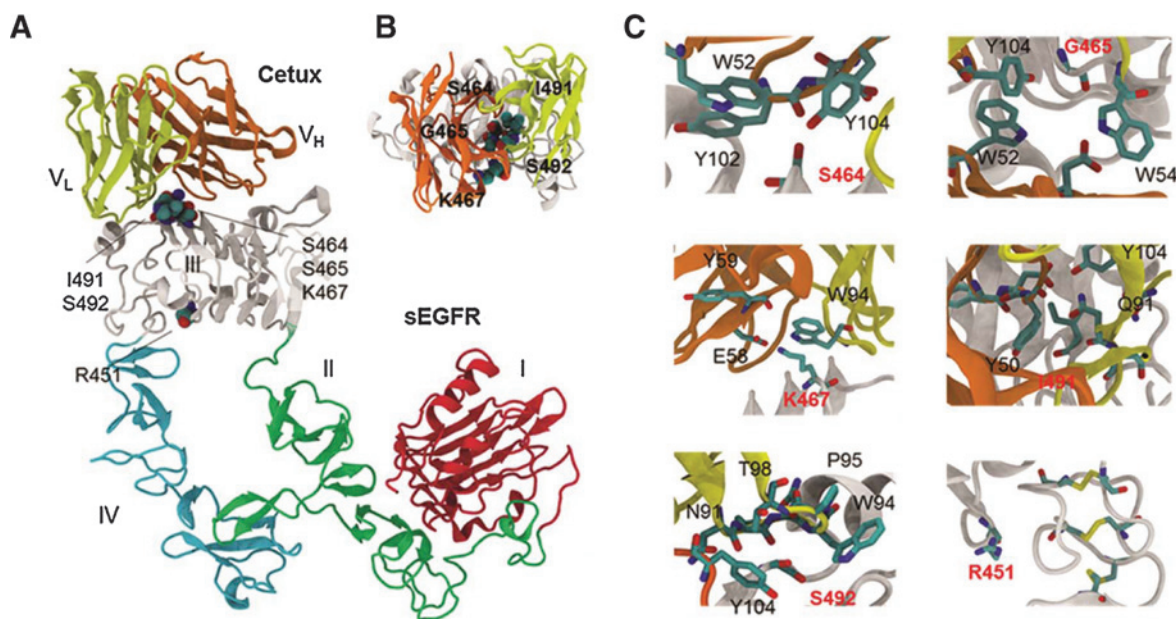
#### EGFR ectodomain mutations and acquired resistance to cetuximab in colorectal cancer cell models

We previously reported that acquisition of resistance in colorectal cancer cells is associated with emergence of *KRAS*-, *BRAF*-, and *NRAS*-activating mutations (6, 9, 12). To discover additional mechanisms of resistance to EGFR blockade, we exploited 5 colorectal cancer cell lines (DiFi, LIM1215, HCA-46, NCIH508, OXCO-2, and CCK81), which are highly sensitive to cetuximab (Supplementary Fig. S2). These cell lines are wild-type for *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* with the exception of NCIH508, which displays the p.E545K *PIK3CA* mutation. Altogether, these cell

models recapitulate the molecular features of tumors from patients with colorectal cancer likely to respond to anti EGFR therapies. For each line, at least five million cells were exposed continuously to cetuximab until resistant populations emerged (Supplementary Fig. S2). To define molecular mechanisms underlying acquisition of resistance, we initially performed Sanger sequencing of genes involved in regulation of the EGFR signaling pathway (*EGFR*, *KRAS*, *BRAF*, *NRAS*, and *PIK3CA*). In accordance with our previous reports, resistant populations often displayed *KRAS*, *BRAF*, and *NRAS* mutations (Table 2; ref. 9). All of these alleles were detected in the resistant cells but not in the corresponding parental population from which they originated. Importantly, in several occasions multiple genetic alterations were concomitantly present in the resistant cell population (Table 2) indicating their polyclonal status. To assess the molecular features of individual clones, we performed limited cell dilutions of LIM1215 and CCK81 as these cell lines are amenable to this procedure. We then subjected single clones to Sanger sequencing for candidate genes (*EGFR*, *KRAS*, *BRAF*, *NRAS*, and *PIK3CA*). Notably, mutation profiling of clones identified three novel *EGFR* variants: S464L, G465R, and I491M (Supplementary Fig. S3). Considering that the resistant derivatives are polyclonal, and in light of the limited sensitivity of the Sanger sequencing method, we postulated that variants present in less than 20% of the cell populations might have remained undetected. To identify mutations present at low frequency, we employed ddPCR, which is known to have a mutant/wild-type sensitivity of 1:20,000. ddPCR probes were designed and individually validated using control mutant DNA to detect *EGFR* variants previously identified in tumor biopsy or cell lines (Supplementary Table S2). This analysis



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**Figure 1.**

Structural analysis of EGFR extracellular domain mutants. A, general overview of the EGFR extracellular domain (sEGFR) bound to the antigen-binding fragment of cetuximab (cetux) as crystallized by Li and colleagues. Four subdomains comprise the sEGFR domain, and domain III contains the six mutations. Mutation R451C occurs at the interface of contact with domain IV. B, mutations I491M, S492R, S464L, G465R, and K467T are located at the surface recognized by cetuximab. A selection of residues located within 5 Å of the site of each mutation are shown. C, S464 (indicated in red, top left) is surrounded by tyrosine residues (Y102 and Y104) which favor an H-bond network. G465 (indicated in red, top right) lies in a highly hydrophobic groove formed by Y104, W54 and W52, K467 (indicated in red, central left) defines a salt bridge with E58. I491 (indicated in red, central right) is located in a hydrophobic environment formed by Q91, Y50, and Y104. S492 (indicated in red, bottom left) is located in an uncharged cavity formed by Y104, W94, P95, T98, and N91. R451 (indicated in red, bottom right) is not located at the cetuximab-sEGFR interface, but closer to the IV domain. Replacement by a cysteine could form a new disulphide bond between domain III and IV.

evidence of their role in driving acquired resistance to EGFR blockade. Of note, the effect of *EGFR* R451C on cetuximab binding was less prominent compared with the other mutants (Fig. 2).

To further characterize the functional properties of the EGFR mutations we performed biochemical studies in cells expressing individual mutations. As expected, cetuximab abrogated ligand-mediated activation of the wild-type receptor, while had no or very limited impact in cells carrying mutated EGFR (S464L, G465R, K467T, I491M, and S492R; Fig. 3). Notably, in R451C-mutant cells, cetuximab was still capable of inhibiting EGFR phosphorylation (Fig. 3).

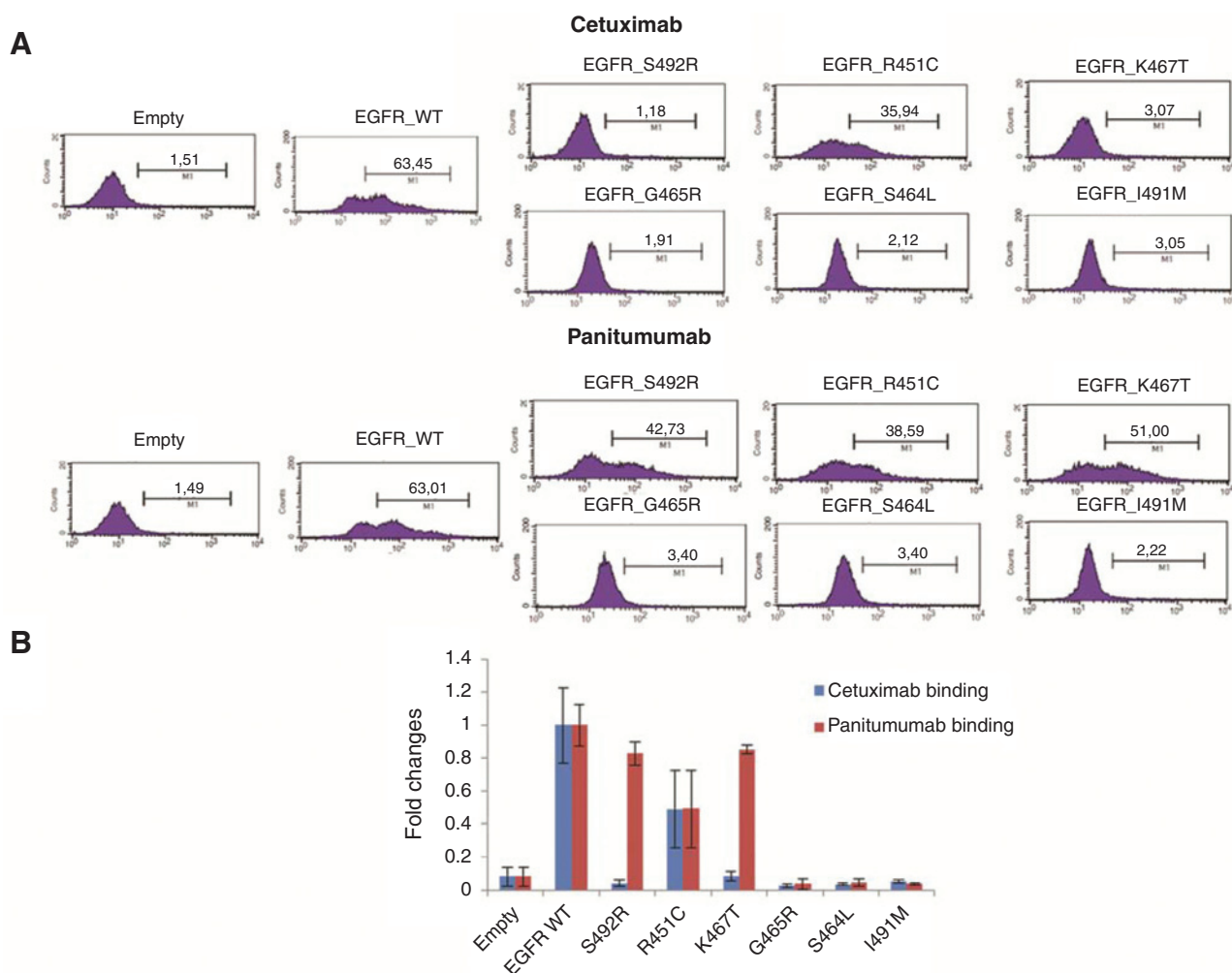
We then examined whether panitumumab, the other anti-EGFR drug approved to treat colorectal cancer, was active in cells overexpressing EGFR mutations. As previously described, S492R-mutant cells efficiently bound to panitumumab (12). We found that the K467T and R451C mutants were to some extent permissive for panitumumab binding, whereas S464L, G465R, and I491M mutants did not bind to this antibody (Fig. 2). Accordingly, biochemical analyses showed that panitumumab prevents EGFR activation in S492R, K467, and R451C mutants (Fig. 3).

## Discussion

We present a comprehensive analysis of mutational changes affecting key members of the EGFR signaling pathway emerging in tumor biopsies of patients treated with cetuximab and in cell models, which acquired resistance to cetuximab *in vitro*. We found that colorectal cancer cells evade EGFR blockade through two main strategies. The main mechanism of resistance involves downstream

pathway reactivation that occurs in 43% of patients' samples and 58.8% of the cells, respectively. The second entails EGFR extracellular domain mutations that were detected in 10.8% of patients' samples and 29% of the cells. Although the specific mutations partially differ among cell lines and patients, they overlap in terms of activated cellular pathways observed in preclinical models and patients samples (22). The cell-based findings may be translated back into the clinic. For example, the EGFR ectodomain alleles, initially discovered in cells, might reasonably also be present in patients who relapse upon EGFR blockade and this could be verified using tissue and liquid biopsies.

The molecular landscape of acquired resistance to EGFR blockade revealed the emergence of multiple point mutations in the ectodomain of EGFR. In particular, we detected two novel EGFR exon 12 mutations (EGFR p.R451C and p.K467T) in 2 patients. The previously reported *EGFR* S492R mutation was detected in 3 of 37 postcetuximab tissue samples (8%), whereas a recent study reports 16% of S492R EGFR mutation detection in 239 postcetuximab plasma samples (11). Such differences may be explained by different sensitivity of the detection techniques as well as the ability of plasma samples to capture the heterogeneity of solid tumors as compared with single biopsies of one tumoral lesion (8, 16, 23). Plasma samples of the patients included in the current study were not systematically collected and therefore we could not analyze the prevalence of EGFR mutations in circulating tumor DNA in the cohort. Accordingly, studies in larger cohorts of patients treated with cetuximab or panitumumab are warranted to define the exact frequency of the other newly identified EGFR ectodomain mutations. Of note, most samples harboring the

**Figure 2.**

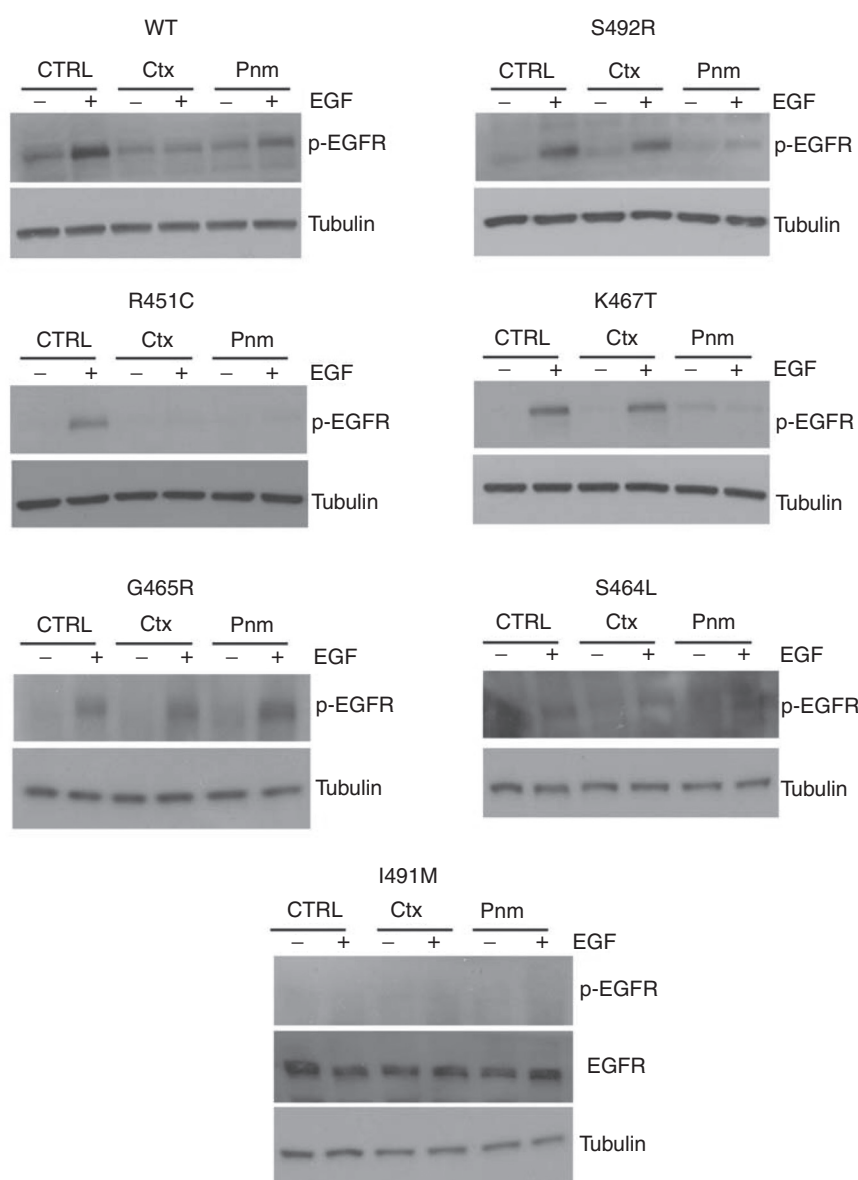
EGFR mutations differentially affect binding to cetuximab and panitumumab. A, NIH 3T3 cells stably expressing wild-type or the indicated EGFR mutations were incubated with cetuximab or panitumumab, and antibody binding was analyzed by flow cytometry using a secondary antibody to human IgG conjugated with phycoerythrin (PE). NIH 3T3 cells expressing the empty vector were used as a negative control (empty). Graphs show results of one representative experiment. B, the percentage of cells binding to the antibody are shown as relative values compared to EGFR wild-type (wt) cells (percentage of EGFR wt cells set to 1) and are mean values of two independent experiments. While cetuximab binding was affected in cells expressing EGFR mutants, panitumumab was able to bind to cells expressing the S492R and K467T EGFR mutation. The R451C mutation had a moderate impact on binding to either cetuximab or panitumumab.

EGFR S492R mutation also exhibited EGFR gene amplification (12), similar to lung cancer tumors harboring the T790M mutation of resistance to tyrosine kinase inhibitors, where the T790M allele appears to be selectively amplified (24). Interestingly, our *in vitro* analysis showed that panitumumab was effective in a subset of EGFR mutants. As the binding epitopes of cetuximab and panitumumab overlap but are not identical, it is foreseeable that mutations arising in EGFR after anti-EGFR treatment will differentially disrupt binding of cetuximab and/or panitumumab to the receptor (25), with relevant clinical implications for the treatment of cetuximab-resistant patients.

Activating mutations in EGFR downstream signaling effectors were the most frequent event in both cell models and patients. Emergence of KRAS and NRAS mutations occurred in 42% of patients, similar to what we have previously reported in tissue samples, but lower than previously reported in plasma samples (6–10). Again, this may be due to the advantage of circulating

DNA in capturing the heterogeneity of solid tumors compared with biopsy of one tumoral lesion (8, 16, 23), indicating that diagnostic tools such as liquid biopsies are required to capture the complexity of the disease. As recently reported, RAS mutations often occurred outside of exon 2 in clinical samples and cell models (26, 27). The finding that codon 61 and 146 KRAS mutations occur more frequently in the acquired resistance setting than in the general colorectal cancer population is worth further studies. Of note, all samples harboring a PIK3CA mutation also displayed other mechanisms of resistance. The role of PIK3CA mutations in driving acquired as well as primary resistance remains controversial and needs to be further characterized. We did not identify emergence of molecular alterations in 20% of patients, suggesting limitations in the sensitivity of the detection technique, tumor heterogeneity, as well as other mechanisms of resistance such as ligand overexpression or c-MET amplification (15, 28, 29).

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**Figure 3.** Ligand-dependent activation of EGFR mutants in the presence of cetuximab and panitumumab. NIH 3T3 cells expressing WT *EGFR* or the indicated *EGFR* mutations were cultured in the presence of cetuximab (Ctx) or panitumumab (Pnm) for 2 hours and stimulated with EGF (5 ng/mL) for 15 minutes. Immunoblotting was performed using antibodies to the indicated proteins.

Importantly, multiple mechanisms of resistance were often present in the samples from relapsed tumors and often displayed more than one molecular alteration. This was also observed in cell lines resistant to cetuximab, strongly suggesting their polyclonal status. This likely reflects the heterogeneity of colorectal cancers and supports the role of circulating DNA to comprehensively characterize the molecular landscape of resistance to EGFR blockade in patients. Activation of EGFR-RAS signaling axis as well as EGFR ectodomain mutations frequently co-occurred. These findings suggest the design of clinical trials that include concomitant inhibition of EGFR downstream signaling together with direct inhibition of the EGFR receptor. Considering that panitumumab seems to be ineffective on a subset of the newly discovered mutations, drugs inhibiting EGFR through different mechanism will also be needed.

Our results have implications for the care of patients as they support the necessity of reassessing the molecular landscape of

tumors after progression to anti-EGFR drugs, which currently is not routinely done in clinical practice. The plasticity of tumor cells and their high capacity of adaptation under selective drug pressure, emphasizes the need for sequential tumor or plasma biopsies to better monitor and personalize treatment.

In summary, our study highlights the importance of reassessing the molecular profile of the overall disease burden longitudinally during therapy, and provides evidence that acquired resistance to anti-EGFR therapy in patients with mCRC arises from the emergence of heterogeneous and overlapping molecular changes. Such complexity converges on two main mechanisms of resistance: activating mutation in EGFR downstream signaling and mutations in EGFR ectodomain that disrupt antibody-receptor binding. In light of these findings, pharmacologic studies combining inhibition of both EGFR and EGFR downstream signaling effectors to bypass cetuximab resistance are warranted.



## Disclosure of Potential Conflicts of Interest

A. Bardelli is a consultant/advisory board member for Biocartis, Horizon Discovery, and Trovogene. C. Montagut is a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** G. Siravegna, A. Martinez, I. Canadas, S. Misale, I. Gonzalez, M. Iglesias, E. Gavilan, S. Hobor, J. Sanchez, A. Dalmases, J. Bellmunt, J. Albanell, C. Montagut

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