









**Table 2.** EGFR pathway mutations in parental and cetuximab-resistant cell lines

Parental cell Lines	Pre-treatment									Resistant cell Lines	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	EGFR exon 12												
												R451C	S464L	G465R	K467T	I491M	S492R							
CCK81										CCK81 R1 Cetux														
										CCK81 R2 Cetux														
										CCK81 R3 Cetux														
DiFi										DiFi R1 Cetux														
										DiFi R2 Cetux														
HCA-46										HCA-46 R1 Cetux														
										HCA-46 R2 Cetux														
LIM1215										LIM1215 R1 Cetux														
										LIM1215 R2 Cetux														
										LIM1215 R3 Cetux														
										LIM1215 R4 Cetux														
										LIM1215 R5 Cetux														
NCIH508										NCIH508 R1 Cetux														
										NCIH508 R2 Cetux														
OXCO-2										OXCO-2 R1 Cetux														
										OXCO-2 R2 Cetux														
										OXCO-2 R3 Cetux														

NOTE: Parental and correspondent cetuximab-resistant derivatives of DiFi, LIM1215, HCA-46, NCIH508, OXCO-2, and CCK81 cell lines were analyzed for *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *EGFR* mutations. Black boxes indicate the presence of the mutations (no specified alleles); in resistant cell lines, mutational analysis for EGFR exon 12 was performed by ddPCR and black boxes indicate which *EGFR* ectodomain alleles emerged.

Abbreviation: Cetux, cetuximab.

unveiled the presence 3 new EGFR variants (S464L, G465R, and I491M) that were not detected by Sanger sequencing in resistant cell populations (Table 2 and Supplementary Table S3). The ddPCR approach could not be performed in tissue samples, as there was no sufficient material available. Overall, the mutational landscape of cell lines with acquired resistance to cetuximab recapitulates the molecular profiles of tumors that relapsed upon cetuximab treatment.

#### Structural model analysis of EGFR ectodomain mutations

To understand how the EGFR ectodomain mutations detected in tumor samples and cell lines could drive resistance to EGFR blockade, we performed computational structure-based analyses. With the exception of R451C, the EGFR mutations are located in the receptor region, which has been shown to interact with cetuximab (Fig. 1A). More specifically, the S464L, G465R, K467T, I491M, and S492R mutations lie in the middle of the surface recognized by the antibody (Fig. 1B) and therefore modifications of this interface have the potential to affect complex formation. Of these five mutations, three appear to disrupt favorable interactions, namely S464L, K467T, and I491M. The polar amino acid S464 is within H-bond distance from the carbonyl backbone of Y102 and the phenolic OH-group of Y104 (Fig. 1C). Replacement by a hydrophobic bulky amino acid as leucine (S464L) has the potential to disrupt the network. Analogously, the positively charged amino acid K467 is involved in a stabilizing salt bridge with E58 (Fig. 1C). Insertion of a polar but neutral amino acid as threonine K467T, although still permitting the H-bond E58, would affect the favorable electrostatic interaction. Residue I491 is a rather large, aliphatic residue located in a hydrophobic cavity mainly formed by aromatic amino acids (Fig. 1C). Although the side chains present similar size, methio-

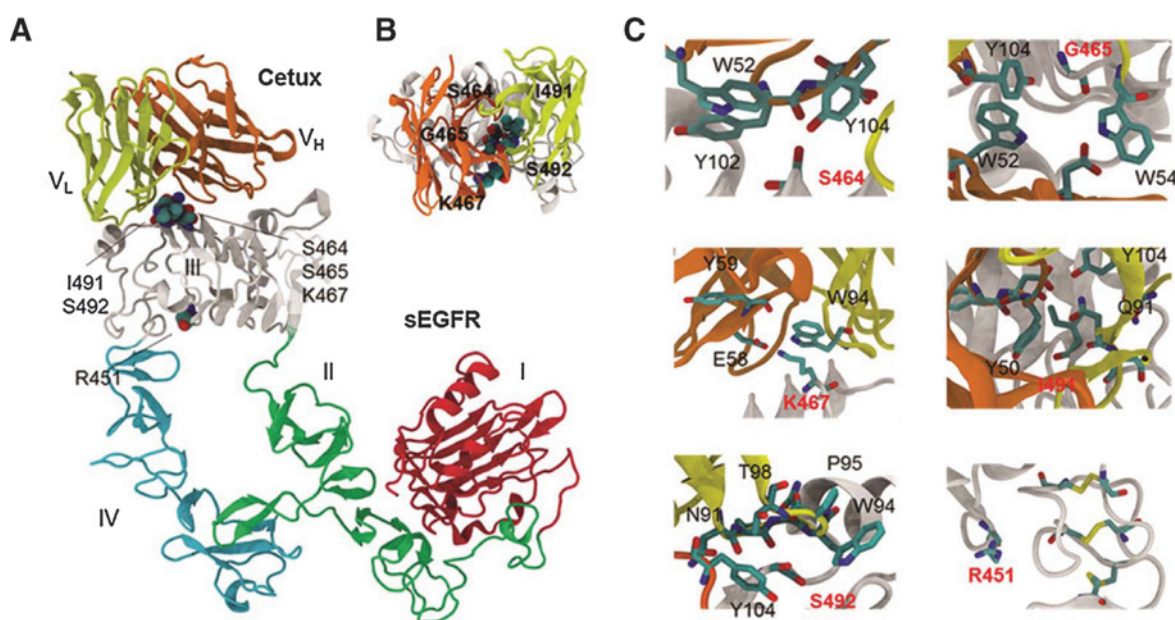
nine (I491M) is a polar amino acid and would be unfavorably located in a nonpolar environment. The other two mutations (G465R and S492R) involve the change from rather small, polar, and uncharged side chain to a large and electrically charged side chain in arginine (Fig. 1C; ref. 13).

R451C is the only mutation not located in the cetuximab-binding site; however, the mutation could lead to critical structural changes. As the domain IV is formed by a sequence of disulphide bonds, which preserve its tertiary structure, the replacement by a cysteine at that position could perturb one of the disulphide bonds in domain III (C475-C462) possibly forming a new one between domains III and IV (C451-C475). We verified that the distance in the crystal structure between these residues (C451-C475, carbon alpha distance 6.8 Å) is compatible with the formation of a disulphide bond. Indeed, a system setup in this new configuration successfully minimizes to a C451-C475 distance of 5.2 Å. Overall, all identified mutations in EGFR (except R451C) were located in the cetuximab-binding epitope.

#### Biochemical and functional analyses of EGFR ectodomain mutations

To experimentally assess the impact of the EGFR ectodomain mutations on the ability of the receptor to interact with cetuximab, we performed forward genetic experiments on the newly discovered mutations. Wild-type and mutant *EGFR* cDNAs were ectopically expressed in NIH 3T3 cells that lack endogenous EGFR. Flow cytometry was used to establish the extent of cetuximab binding to cells expressing the mutants; wild-type *EGFR* and S492R served as positive and negative controls, respectively. These experiments clearly showed that the newly discovered EGFR K467T, R451C, S464L, G465R, and I491M mutations were not permissive for binding to cetuximab thus providing functional

Arena et al.

**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).

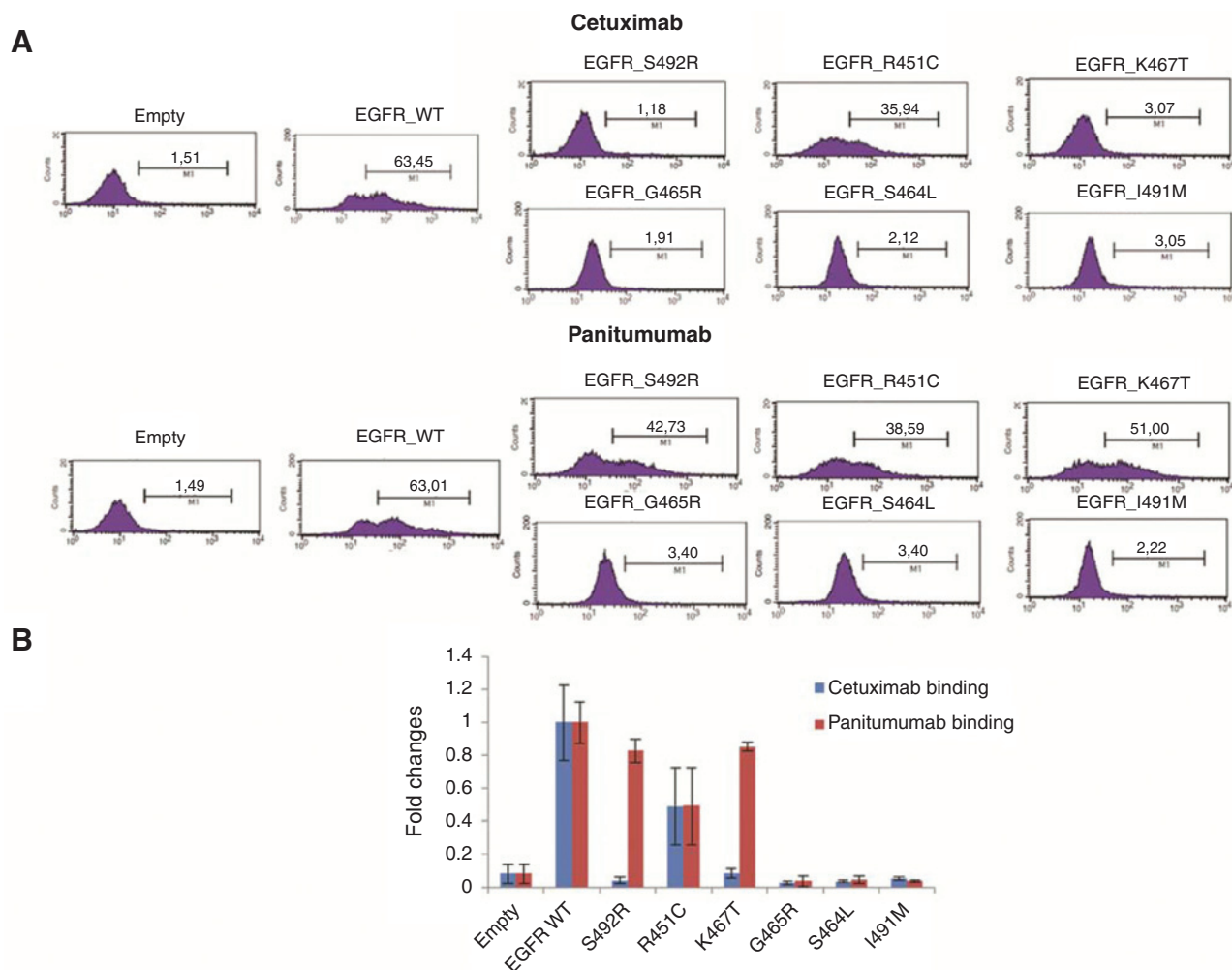
We then examined whether panitumumab, the other anti-EGFR drug approved to treat colorectal cancer, was active in cells overexpressing EGFR mutations. As previously described, S492R-mutant cells efficiently bound to panitumumab (12). We found that the K467T and R451C mutants were to some extent permissive 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.**

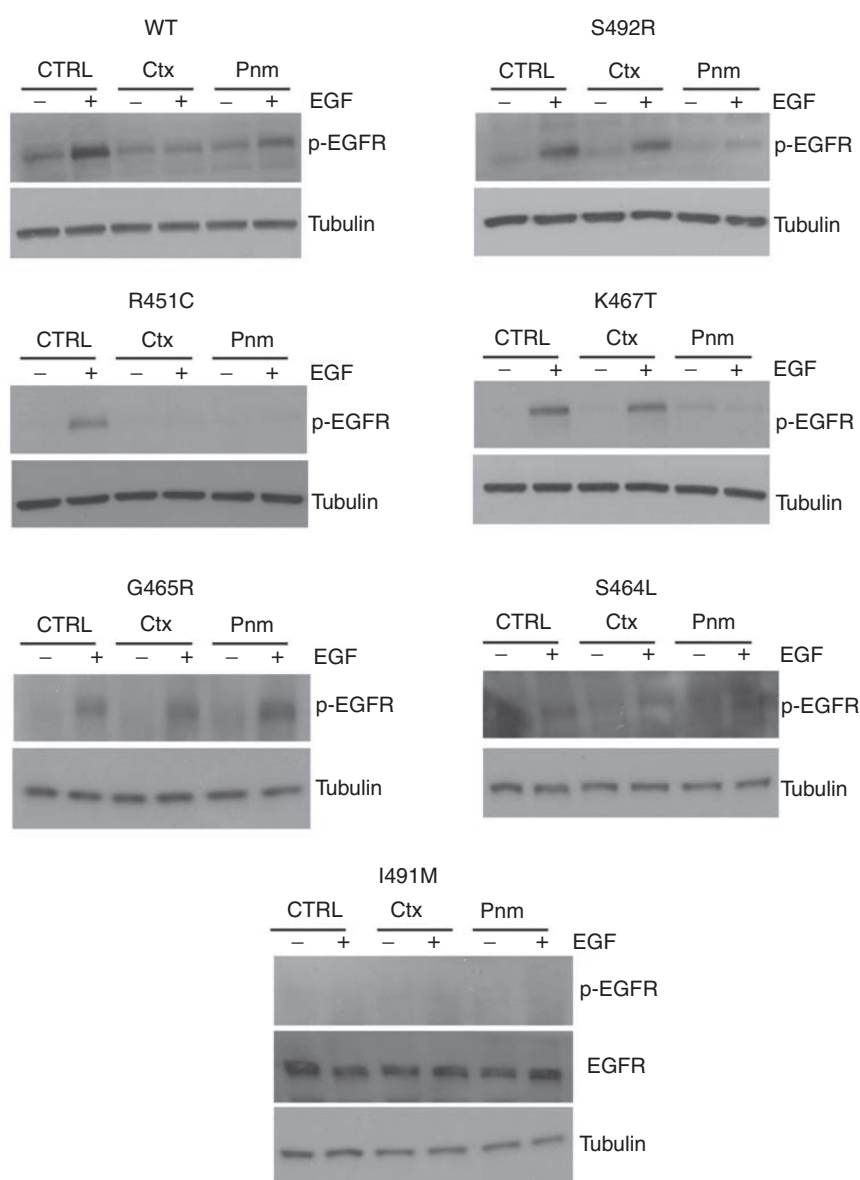
EGFR mutations differentially affect binding to cetuximab and panitumumab. A, NIH 3T3 cells stably expressing wild-type or the indicated EGFR mutations were incubated with cetuximab or panitumumab, and antibody binding was analyzed by flow cytometry using a secondary antibody to human IgG conjugated 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).

Arena et al.



**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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Arena et al.

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