Emergence of Multiple EGFR Extracellular Mutations during Cetuximab Treatment in Colorectal Cancer

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Abstract

Purpose: Patients with colorectal cancer who respond to the anti-EGFR antibody cetuximab often develop resistance within several months of initiating therapy. To design new lines of treatment, the molecular landscape of resistant tumors must be ascertained. We investigated the role of mutations in the EGFR signaling axis on the acquisition of resistance to cetuximab in patients and cellular models.

Experimental Design: Tissue samples were obtained from 37 patients with colorectal cancer who became refractory to cetuximab. Colorectal cancer cells sensitive to cetuximab were treated until resistant derivatives emerged. Mutational profiling of biopsies and cell lines was performed. Structural modeling and functional analyses were performed to causally associate the alleles to resistance.

Results: The genetic profile of tumor specimens obtained after cetuximab treatment revealed the emergence of a complex pattern of mutations in EGFR, KRAS, NRAS, BRAF, and PIK3CA genes, including two novel EGFR ectodomain mutations (R451C and K467T). Mutational profiling of cetuximab-resistant cells recapitulated the molecular landscape observed in clinical samples and revealed three additional EGFR alleles: S464L, G465R, and I491M. Structurally, these mutations are located in the cetuximab-binding region, except for the R451C mutant. Functionally, EGFR ectodomain mutations prevent binding to cetuximab but a subset is permissive for interaction with panitumumab.

Conclusions: Colorectal tumors evade EGFR blockade by constitutive activation of downstream signaling effectors and through mutations affecting receptor–antibody binding. Both mechanisms of resistance may occur concomitantly. Our data have implications for designing additional lines of therapy for patients with colorectal cancer who relapse upon treatment with anti-EGFR antibodies.

Introduction

Monoclonal antibodies (mAb) directed against the EGFR, cetuximab, and panitumumab, provide significant survival benefit to patients with RAS wild-type metastatic colorectal cancer (mCRC) and are now standard components of treatment regimens for these patients, either alone or in combination with chemotherapy. However, the duration of this response is only transient and does not last more than 3 to 12 months, after which secondary resistance occurs (1–5). Definition of the molecular changes underlying acquired resistance to anti-EGFR antibodies is needed to improve clinical benefit and devise further lines of treatment. Molecular profiling of patient samples and preclinical models has previously defined a number of mechanisms of secondary resistance. The most common event is the emergence of KRAS and NRAS mutations which occurs in approximately 50% to 80% of the cases (6–10). Other escape routes include acquisition of the S492R mutation in the ectodomain of EGFR (10–13), as well as amplification of tyrosine kinase receptor genes HER2 or MET (14, 15). Interestingly, recent data indicate the coexistence of several molecular mechanisms of secondary resistance within an individual patient (6–10, 12, 16).
To identify yet unknown molecular events leading to anti-EGFR treatment failure, we investigated the role of mutations in the EGFR signaling pathway on the acquisition of resistance to cetuximab in patients with colorectal cancer and cellular models. Our findings suggest that we have yet to fully characterize the complete spectrum of genetic variants through which colorectal tumors evade EGFR blockade. The data presented here will help design additional therapeutic strategies to circumvent resistance of patients with colorectal cancer who receive EGFR-targeted antibodies.

**Materials and Methods**

**Tumor samples and patients**

All mCRC consenting patients treated with anti-EGFR mAb at Hospital del Mar (Barcelona, Spain) between January 2010 and June 2013 were included in this study. In the analysis, we only included patients who had good quality paired pre- and post-treatment biopsies and who acquired resistance to anti-EGFR-based therapy defined as progression disease following (i) complete response or partial response or (ii) stable disease for more than 16 weeks. Response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST; ref. 17). Our study included re-biopsy following treatment failure in patients who consented to this extra procedure. Re-biopsies at the time of progression were obtained from the most accessible lesion with less potential risk of related complications for the patient according to ethical considerations. Pretreatment sample was obtained from the regular diagnosis procedure. In this study, we included 9 cases (patients #21–#28 and patient #36) that had been previously assessed for EGFR S492R, KRAS exon 2, BRAF V600E, and PIK3CA mutations by Sanger sequencing (12) and that in the current work were analyzed for a broader panel of mutations as specified below using more sensitive sequencing technologies. Biologic samples were obtained from Parc de Salut Mar Biobank (MARBiobanc). This study was approved by the local Ethics Board (MARBiobanc). This study was approved by the local Ethics Board (MARBiobanc).

**Mutational analysis in tissue samples**

DNA extraction from tumoral samples was performed as previously described (12). Mutational analysis of KRAS (exons 2, 3, and 4), BRAF (exon 15), NRAS (exons 2 and 3), PIK3CA (exons 9 and 20), and EGFR (exon 12) was performed by Sanger sequencing using BigDye v3.1 (Applied Biosystems) following manufacturer’s instructions and analyzed on a 3500Dx Genetic Analyzer (Applied Biosystems). KRAS (exons 3 and 4) and NRAS (exons 2, 3, and 4) were also analyzed by pyrosequencing (Qiagen) and KRAS (exon 2) was also assessed by Therascreen real-time PCR (Qiagen) following the manufacturer’s instructions. All cases were also screened by pyrosequencing using a next generation sequencing (NGS) 454 GS Junior platform (Roche Applied Science). Processed and quality-filtered reads were analyzed using the GS Amplicon Variant Analyzer software version 2.5p1 (Roche). Mutations detected by NGS were confirmed by competitive allele-specific TaqMan PCR (CAST-PCR, Applied Biosystems) when specific assays were available.

**Cell culture and generation of resistant cells**

DiFi cells were cultured in F12 medium (Invitrogen) supplemented with 5% FBS; OXCO-2 cells were cultured in Iscove medium (Invitrogen) supplemented with 5% FBS; LIM1215 cells were cultured in RPMI1640 medium (Invitrogen) supplemented with 5% FBS and insulin (1 μg/mL); NCH508 cells were cultured in RPMI1640 medium (Invitrogen) supplemented with 5% FBS; HCA-46 cells were cultured in DMEM (Invitrogen) supplemented with 5% FBS and CCK81 cells were cultured in MEM (Invitrogen) supplemented with 5% FBS. All media also contained 2 mmol/L L-glutamine and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), and cells were grown in a 37°C and 5% CO₂ air incubator. The DiFi and OXCO-2 cell lines were a kind gift from Dr. J. Baselga in November 2004 (Oncology Department of Vall d’Hebron University Hospital, Barcelona, Spain) and Dr V. Cerundolo in March 2010 (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom), respectively. The LIM1215 parental cell line has been described previously (18) and was obtained from Prof. Robert Whitehead (Vanderbilt University, Nashville, TN) with permission from the Ludwig Institute for Cancer Research (Zurich, Switzerland). The NCH508 cell line was purchased from ATCC (LGC Standards S.r.l.). HCA-46 cell lines were obtained from European Collection of Animal Cell Cultures (distributed by Sigma-Aldrich Stl). CCK81 cell line was obtained from Health Science Research Resources Bank. The identity of each cell line was tested and authenticated by Cell ID System and by Gene Print 10 System (Promega), through short tandem repeats (STR) at 10 different loci (D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, TPOX, CSF1PO, and amelogenin). Amplicons from multiplex PCRs were separated by capillary electrophoresis (3730 DNA Analyzer, Applied Biosystems) and analyzed using GeneMapperID software from Life Technologies. Resulting cell line STR profiles were cross-compared and matched with the available STR from ATCC, ECACC, and CellBank Australia repositories online databases. All cell lines were tested and resulted negative for mycoplasma contamination with Venor GeM Classic Kit (Minerva Biolabs).

Generation of resistant cells utilized in this article has already been previously described (6, 9). CCK81 cetuximab-resistant derivatives were obtained by increasing the cetuximab dosage stepwise from 680 nmol/L to 1.4 μmol/L during the course of 6 months.

**Translational Relevance**

The anti-EGFR monoclonal antibody cetuximab is effective for the treatment of patients with “RAS” wild-type metastatic colorectal cancer (mCRC). Unfortunately, responses are transient, and most patients develop acquired resistance. In the current study, we analyzed the genetic profile of clinical specimens and preclinical models of acquired resistance to cetuximab, revealing a complex pattern of mutations in both EGFR and its downstream effectors, KRAS, NRAS, BRAF, and PIK3CA. We discovered five novel point mutations in the ectodomain of EGFR, which confer resistance to cetuximab. Of note, only a subset of these EGFR variants remains sensitive to panitumumab. Accordingly, the identification of patient-specific mechanisms of resistance to EGFR blockade will be paramount to design additional lines of therapy.
Mutational analysis in cell lines

Genomic DNA samples were extracted by Wizard SV Genomic DNA Purification System (Promega). For Sanger sequencing, all samples were subjected to automated sequencing by ABI PRISM 3730 (Applied Biosystems). Primer sequences are listed elsewhere (6, 9). The following genes and exons were analyzed: KRAS (exons 2, 3, and 4), NRAS (exons 2 and 3), PIK3CA (exons 9 and 20), BRAF (exon 15), EGFR (exon 12). All mutations were confirmed twice, starting from independent PCR reactions.

Drug assays

Cetuximab was obtained from the Pharmacy at Niguarda Ca’ Granda Hospital, Milan, Italy. Cell lines were seeded in 100 µL medium at the following densities (2 × 10^4 for DFi, 1.5 × 10^4 for LM1215, HCA-46, NCI-H508, and OXCO-2, 3 × 10^4 for CCR81) in 96-well culture plates. After serial dilutions, cetuximab in serum-free medium was added to cells, and medium-only wells were included as controls. Plates were incubated at 37°C in 5% CO2 for 6 days, after which cell viability was assessed by ATP content using the CellTiter-Glo Luminescent Assay (Promega).

DNA constructs and mutagenesis

The pLX301-EGFR WT construct was a generous gift from Dr. C. Sun and Prof R. Bernards (NKI, Amsterdam, the Netherlands). EGFR mutants containing the 6 point mutations (R451C, S464L, E411K, D494H, P738L, and G726V) in exon 18 were cloned into the pLX301-EGFR WT plasmid as the template DNA. The presence of mutations was confirmed by DNA sequencing.

Droplet digital PCR

Isolated gDNA was amplified using ddPCR Supermix for Probes (Bio-Rad) using KRAS, NRAS, BRAF, and EGFR assay (PrimePCR ddPCR Mutation Assay, Bio-Rad and custom designed). Droplet digital PCR (ddPCR) was performed according to manufacturer’s protocol and the results reported as percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild-type) DNA alleles. Eight to 10 µL of DNA template was added to 10 µL of ddPCR Supermix for Probes (Bio-Rad) and 2 µL of the primer/microbe mixture. This 20 µL sample was added to 70 µL of Droplet Generation Oil for Probes (Bio-Rad) and used for droplet generation. Droplets were then thermal cycled with the following conditions: 5 minutes at 95°C, 40 cycles of 94°C for 30 seconds, 55°C for 1 minute followed by 98°C for 10 minutes (ramp rate 2°C/sec). Samples were then transferred to a QX200 Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes. Gating was performed on the basis of positive and negative controls, and mutant populations were identified. Fractional abundances of the mutant DNA in the wild-type DNA background were calculated for each sample using QuantaSoft software (Bio-Rad). Multiple replicates (minimum of four) were performed for each sample; ddPCR analysis of normal control gDNA from cell lines and no DNA template (water) controls were performed in parallel with all the samples, including again multiple replicates as a contamination-free control.

EGFR probes and primers sequences are available upon request.

Molecular simulations

Input coordinates for the structure of cetuximab bound to wild-type sEGFR (extracellular part) were taken from PDB:1YY9 (19). The system was parameterized using the Amber12 force field (20) and solvated and neutralized in a TIP3P water box. Energy minimization was conducted under NPT conditions at 1 atm, 298K, and a cutoff of 9 Å, with rigid bonds and PME for long-range electrostatics. Potential energy minimization was run for 2 ps. During minimization, the heavy protein atoms were not restrained. The simulation was run using ACEMD (21) on a local Graphics Processing Unit (GPU)-equipped workstation.

Flow cytometry

To measure cetuximab and panitumumab binding to cells expressing mutant EGFR, we harvested by trypsinization and washed the cells twice with PBS. We incubated the cells with Fc blocking solution for 15 minutes on ice to block nonspecific Fc binding of immunoglobulins. We then washed the cells and incubated them with the monoclonal antibodies for EGFR binding during 30 minutes on ice. To visualize the primary antibody a goat anti-human IgG phycoerythrin-conjugated (Invitrogen) was used as a secondary antibody. EGFR binding was analyzed using the FACScan flow Cytometer.

Protein detection

We subjected total cell lysates to Western blot analysis as previously reported (12). The phospho-EGFR antibody (Y1068) was purchased from Cell Signaling Technology.

Results

Emergence of EGFR ectodomain mutations in patients treated with cetuximab

Thirty-seven consecutive patients with mCRC who had acquired resistance to cetuximab after an initial response to the treatment and had good quality paired pre- and posttreatment specimens were included in this study. Tumor biopsy obtained during the regular diagnosis procedure was used as the pretreatment sample. In most cases, this sample was obtained from the primary tumor during routine colonoscopy. Biopsies at progression were taken from liver (21 samples), lung (6), bone (2), peritoneum (3), colon-rectum (3), retroperitoneal lymph node (1), and subcutaneous node (1) with ultrasound guidance, CT scan guidance, or colonoscopy in the case of colon-rectum lesions. There were no major biopsy-related complications. Clinical characteristics of the patients are showed in Supplementary Table S1.

All pretreatment biopsies were screened for mutations in KRAS, NRAS, and BRAF by Sanger and pyrosequencing as part of routine clinical practice at Hospital del Mar (Barcelona, Spain). Mutations in PIK3CA and EGFR were also assessed as part of this study. All pretreatment biopsies were wild-type except for three samples that harbored mutations in PIK3CA. As PIK3CA mutations do not preclude response to anti-EGFR therapy, these patients were treated with cetuximab-based regimens. Posttreatment tissue samples were analyzed for the same mutations using the same sequencing platforms. In total, we detected the emergence of 31 mutations in posttreatment biopsies from 20 patients (Table 1). The additional 17 posttreatment samples did not reveal variants in any of the genes we analyzed. In 7 cases, mutations in different genes were detected in the same tissue sample (median of detected mutations within the same specimen 2, range 1–5; Table 1).

Acquired mutations were found in NRAS (9 events) and KRAS genes (8 events) followed by mutations in PIK3CA (6 events) and BRAF (3 events). Interestingly, mutations in RAS frequently occurred in exons 3–4 (67% of NRAS and 50% of KRAS...
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, NCH508, 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 NCH508, 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 K467T (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 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

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
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<tr>
<td></td>
<td>KRAS exon 2</td>
<td>KRAS exon 3</td>
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</table>

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.
unveiled the presence of 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. Of these five mutations, three appear to disrupt favorable interactions, namely S464L, K467T, and I491M.

### 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

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

<table>
<thead>
<tr>
<th>Parental cell lines</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
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<tbody>
<tr>
<td></td>
<td>Resistant cell lines</td>
<td>EGFR exon 12</td>
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<tr>
<td></td>
<td>KRAS exon 2</td>
<td>KRAS exon 3</td>
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<tr>
<td>CCK81</td>
<td>CCK81 R1 Cetux</td>
<td>CCK81 R2 Cetux</td>
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<tr>
<td>DIFI</td>
<td>DIFI R1 Cetux</td>
<td>DIFI R2 Cetux</td>
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<tr>
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<td>HCA-46 R1 Cetux</td>
<td>HCA-46 R2 Cetux</td>
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<tr>
<td>LIM1215</td>
<td>LIM1215 R1 Cetux</td>
<td>LIM1215 R2 Cetux</td>
</tr>
<tr>
<td>NCIH508</td>
<td>NCIH508 R1 Cetux</td>
<td>NCIH508 R2 Cetux</td>
</tr>
<tr>
<td>OXCO-2</td>
<td>OXCO-2 R1 Cetux</td>
<td>OXCO-2 R2 Cetux</td>
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</tbody>
</table>

**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.
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
EGFR S492R mutation also exhibited EGFR gene amplification similar to lung cancer tumors harboring the T790M mutation of resistance to tyrosine kinase inhibitors, where the T790M allele appears to be selectively amplified. 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, 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. 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, 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. 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.

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. 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.
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 mechanisms 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 Trovagene. C. Montagut is a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Acknowledgments

The authors thank Dr. Salvatore Siena, Dr. Andrea Sartore Bianchi, and members of the Laboratory of Molecular Genetics for critical reading and editing of this article. The authors also thank Fundacio Cellex (Barcelona) for a generous donation to the Hospital del Mar Medical Oncology Service.

Grant Support

This work was supported by RD12/0063/0051, P11/00989, P11/00680, PT13/0010/0005, 2014 SGR 567 and 2014 SGR 740 grants and by the Xarxa de Banc de Tumors de Catalunya (to C. Montagut). The European Community’s Seventh Framework Programme under grant agreement no 259915 COLHERES (to A. Bardelli), Associazione Italiana per la Ricerca sul Cancro (AIRC) IG grant no. 12812 (to A. Bardelli); AIRC MFAG no. 11349 (to F. Di Nicolantonio); grant DFarmacogenomica—5 per mille 2009 MIUR—Fondazione Piemontese per la Ricerca sul Cancro—ONLUS (to F. Di Nicolantonio); AIRC 2010 Special Program Molecular Clinical Oncology 5 per mille, project no. 9970 (to A. Bardelli); FP7C 5 per mille 2010 and 2011 Ministero della Salute (to A. Bardelli); Ministero dell’Istruzione, dell’Università e della Ricerca, progetto PRIN 2010-2011 (to A. Bardelli). J. Albanell is a recipient of intensification program ISCIII/FEDER.

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Received November 5, 2014; revised December 19, 2014; accepted January 8, 2015, published OnlineFirst January 26, 2015.
Emergence of Multiple EGFR Extracellular Mutations during Cetuximab Treatment in Colorectal Cancer

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