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Abstract

The anti–epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab are used to treat RAS wild-type colorectal cancers (CRCs), but their efficacy is limited by the emergence of acquired drug resistance. After EGFR blockade, about 20% of CRCs develop mutations in the EGFR extracellular domain (ECD) that impair antibody binding and are associated with clinical relapse. We hypothesized that EGFR ECD–resistant variants could be targeted by the recently developed oligoclonal antibody MM-151 that binds multiple regions of the EGFR ECD. MM-151 inhibits EGFR signaling and cell growth in preclinical models, including patient-derived cells carrying mutant EGFR. Upon MM-151 treatment, EGFR ECD mutations decline in circulating cell-free tumor DNA (ctDNA) of CRC patients who previously developed resistance to EGFR blockade. These data provide molecular rationale for the clinical use of MM-151 in patients who become resistant to cetuximab or panitumumab as a result of EGFR ECD mutations.

INTRODUCTION

The anti–epidermal growth factor receptor (EGFR) monoclonal antibodies (mAbs) cetuximab and panitumumab are effective in a subset of RAS/BRAF wild-type metastatic colorectal cancers (mCRCs). However, the onset of secondary resistance limits their clinical benefit (1, 2). Previous studies indicated that the emergence of genetic alterations involving EGFR downstream effectors (KRAS, NRAS, and BRAF) (3–6) or activation of parallel receptor tyrosine kinase (RTK) pathways (HER2 and MET) (7–9) can confer acquired resistance to cetuximab or panitumumab. We and others further reported that mutations in the EGFRextracellular domain (ECD) (p.S492R, p.R451C, p.S464L, p.G465R, p.K467T, and p.I491M) also mediate secondary resistance to EGFR blockade in colorectal cancer (10–13). Notably, most of these variants are resistant to both cetuximab and panitumumab (11).

This work was initiated on the hypothesis that colorectal tumors displaying EGFR ECD mutations may retain dependence on EGFR beyond progression on previous treatment with anti-EGFR mAbs. If this assumption proved correct, mCRC patients who become resistant to cetuximab or panitumumab due to the emergence of EGFR ECD

mutations may still benefit from further lines of treatment with EGFR inhibitors. We reasoned that it may be possible to target cells carrying ECD mutations with drugs that bind to different epitopes located in the EGFR ECD. The use of mixtures of mAbs recognizing distinct epitopes of RTKs has proven effective in preclinical and clinical experimentation, as exemplified by the combination of trastuzumab and pertuzumab in HER2-overexpressing breast cancer (14–19). Compared to cetuximab or panitumumab alone, EGFR-targeted antibody mixtures induce more profound receptor endocytosis and suppression, which result in enhanced anticancer effects in mouse models (20, 21). These observations prompted the design and development of mAb combinations targeting EGFR on multiple, nonoverlapping epitopes. Among these, MM-151 is a third-generation EGFR inhibitor consisting of three fully human immunoglobulin G1 antibodies that simultaneously engage distinct, non-overlapping epitopes on EGFR (22). MM-151 has demonstrated superiority to currently approved and investigational mAbs in preclinical models, displaying improvements in EGFR pathway inhibition and downstream signaling, as well as enhanced down-regulation of the EGFR and engagement of innate immune responses (22). Notably, MM-151 targets regions of the EGFR distinct from those affected by ECD mutations (Fig. 1A). Here, we tested the hypothesis that colorectal cancers (CRCs) that develop resistance to cetuximab or panitumumab through EGFR ECD mutations might be sensitive to EGFR blockade by MM-151.

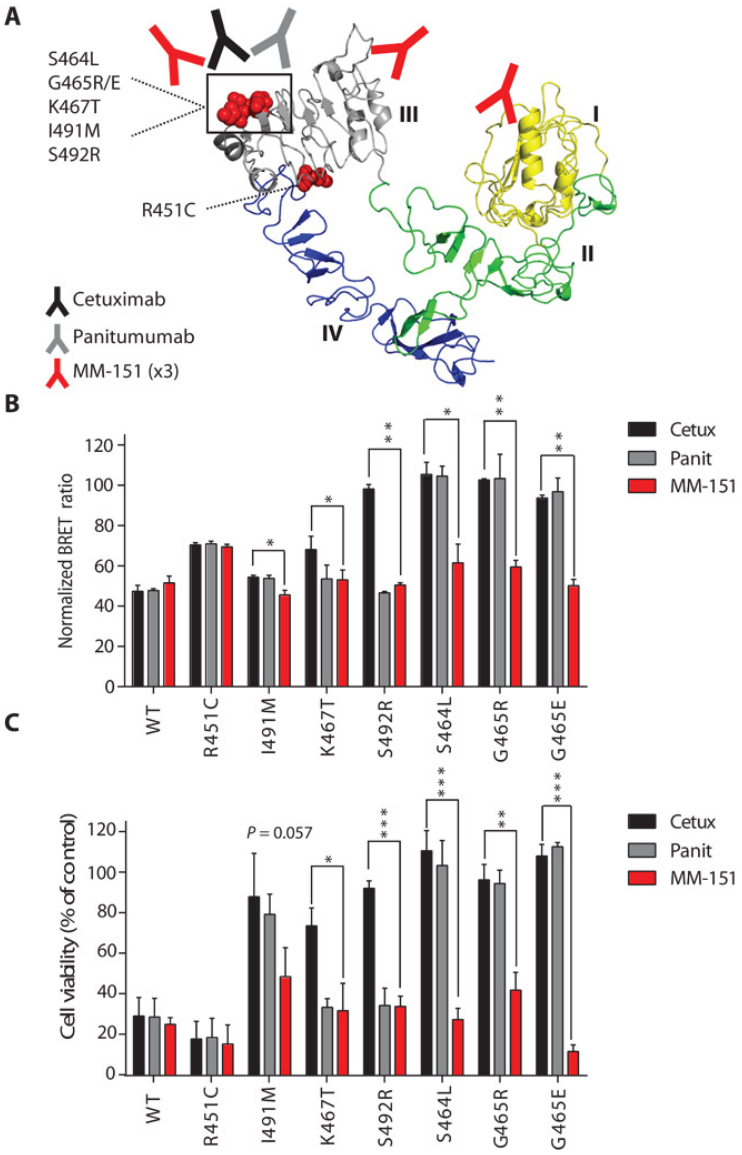

Fig. 1.MM-151 engages three epitopes on the EGFR extracellular region and binds all EGFR ectodomain mutants.

(A) Schematic representation of the four EGFR ECDs derived from Protein Data Bank (PDB) structure "1NQL" (42). Highlighted in red are six amino acid positions identified as mutated in cetuximab-resistant tumors (EGFR R451C, S464L, G465E/R, K467T, I491M, and S492R). Approximate binding sites for cetuximab, panitumumab, and MM-151 are indicated on the basis of published data (22, 47, 48). (B) NanoLuc drug displacement assay showing ligand antagonism activities of anti-EGFR drugs on cells expressing EGFR ectodomain mutants. HEK-293 cells were transiently transfected with plasmids expressing the indicated NanoLuc-EGFR mutants and then treated with cetuximab (5 μg/ml), panitumumab, or MM-151 and HaloTag-EGF (tracer) at a concentration of 18 ng/ml for 30 min. The BRET ratio was normalized to the ligand-only control. Each experiment was repeated at least two times with duplicate replicates, with means ± SD indicated. (C) Cell proliferation assay performed on a panel of LIM1215 CRC cells engineered to express the indicated EGFR ectodomain mutants. Cells were treated for 6 days with increasing concentrations of cetuximab (black bars), panitumumab (gray bars), or MM-151 (red bars), and cell viability was measured by the adenosine triphosphate (ATP) assay. Results are normalized to untreated control and shown as a bar plot for a drug concentration of 5 μg/ml and as a drug titration series in fig. S3. The experiment was repeated three times, with mean ± SD indicated. Cetux, cetuximab; Panit, panitumumab; WT, wild type. Statistical differences were calculated by two-tailed unpaired Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001. Detailed data and P values are provided in tables S1 and S2.

RESULTS

Impact of EGFR ECD mutations on ligand-receptor binding

We previously identified six distinct EGFR ECD mutations in CRC cells and patients who had become resistant to cetuximab or panitumumab (10, 11). In addition, we recently detected another ECD mutation (p.G465E) in a cetuximab-resistant cell line (HCA-46 R5), which we established by culturing drug-sensitive parental HCA-46 cells in the presence of the antibody (fig. S1).

A key question is whether EGFR variants that emerge upon cetuximab or panitumumab exposure remain capable of ligand-mediated intracellular signaling. To study whether the mutant EGFRs can interact with the ligand, epidermal growth factor (EGF), we designed an assay that detects bioluminescent resonance energy transfer (BRET) from a luminescent protein donor to a fluorescent protein acceptor. To this end, we used a receptor fused with nanoluciferase at the N-terminal domain (NanoLuc-EGFR) as the energy donor and a fluorescently labeled HaloTag fusion ligand (HaloTag-EGF) as the energy acceptor. NanoLuc-EGFR vectors corresponding to each of the ECD variants (p.R451C, p.S464L, p.G465R, p.G465E, p.K467T, p.I491M, and p.S492R) were generated and expressed in human embryonic kidney (HEK)–293 cells. A vector encoding the wild-type receptor (pNanoLuc-EGFR WT) served as a control. First, we measured the ability of HaloTag-EGF to bind wild-type or mutant receptors in the presence of competing high doses of unlabeled EGF. We found that all mutants can interact with EGF in a dose-dependent manner (fig. S2). The EGFR R451C mutant was less efficient than the others at binding EGF. We speculate that this may be associated with a low-affinity binding conformation (due to the presence of the cysteine residue), which might form disulfide bonds with domain IV. The finding that EGFRs carrying ECD mutations remain capable of binding EGF is of relevance because it suggests that cells that become resistant to anti-EGFR antibodies do so while maintaining reliance on EGFR-mediated signaling.

Impact of EGFR ECD mutations on antibody-receptor binding

We next assessed whether anti-EGFR antibodies were capable of interfering with binding of EGF to the ECD mutant receptor. The NanoBRET assay was performed in the presence of cetuximab, panitumumab, and MM-151 to measure their ability to displace the ligand EGF from the wild-type or mutant EGFR proteins. We observed that only MM-151 was able to bind all EGFR ECD mutants to an extent comparable with EGFR WT (Fig. 1B and table S1).

Effects of anti-EGFR blockade on LIM1215 cells overexpressing EGFR ECD mutants

To measure the impact of EGFR ECD mutations on cell proliferation and survival in the presence of EGFR-targeted mAbs, we took advantage of the CRC cell line LIM1215, which is highly sensitive to cetuximab and panitumumab (3, 11). We used site-directed mutagenesis to develop lentiviral expression vectors for wild-type EGFR (control) and seven EGFR ECD variants (p.R451C, p.S464L, p.G465R, p.G465E, p.K467T, p.I491M, and p.S492R) and generated a LIM1215 cell line panel expressing individual mutations. To assess the effect of cetuximab, panitumumab, and MM-151 on individual ECD mutations, we performed short-term cell proliferation assays (Fig. 1C, table S2, and fig. S3) and signaling pathway analysis (Fig. 2) on the LIM1215 cell line panel.

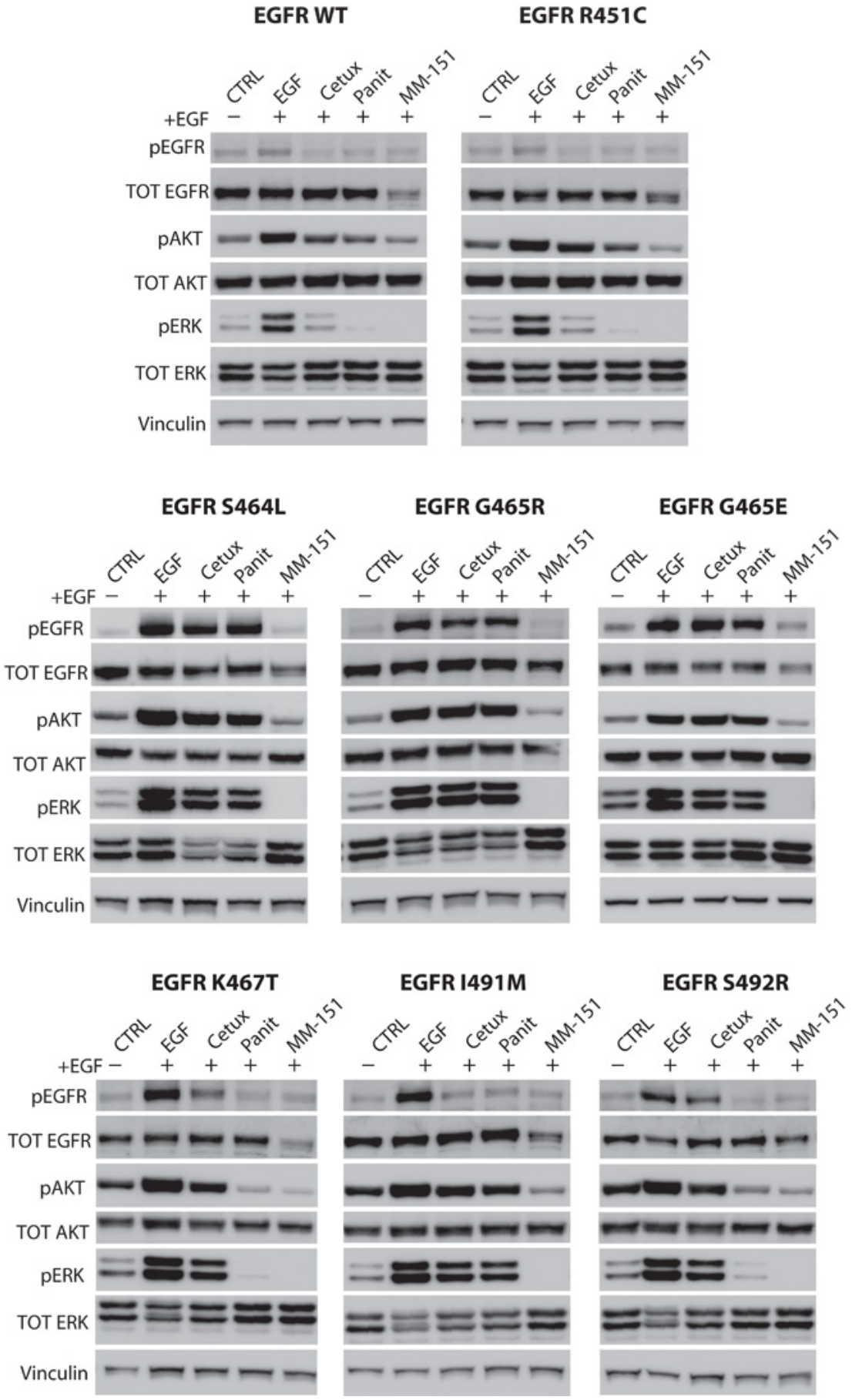

Fig. 2. MM-151 inhibits EGFR signaling in cells expressing ECD mutations.

LIM1215 cells engineered to express the indicated EGFR ectodomain mutants were cultured in the presence of cetuximab (Cetux), panitumumab (Panit), or MM-151 for 2 hours and stimulated with EGF (5 ng/ml) for 15 min. Cell extracts were immunoblotted to detect the indicated total or phosphorylated proteins and vinculin (loading control). CTRL, control.

We found that p.R451C-expressing cells were still sensitive to cetuximab treatment, thus underlining again the peculiarity of this mutant, which remains sensitive to cetuximab or panitumumab (11). Cells expressing EGFR p.S464L, p.G465R, p.G465E, and p.I491M showed cross-resistance to panitumumab, whereas the remaining genotypes (EGFR p.K467T and p.S492R) were sensitive to panitumumab treatment (Fig. 1C and fig. S3). To provide a mechanistic context for the differences in the cell growth inhibition assays, we performed biochemical analysis of the EGFR/ERK (extracellular signal–regulated kinase)/AKT signaling pathway. After MM-151 treatment, a reduction in the amounts of phospho-EGFR and downstream ERK and AKT effector proteins was observed in cells expressing the EGFR ECD mutants that were insensitive to cetuximab or panitumumab in the proliferation assay (Fig. 2).

Whereas cetuximab and panitumumab were effective only on a subset of the resistant ECD mutations, all ECD mutants were markedly sensitive to MM-151 (Fig. 2). These results show that the oligoclonal antibody mixture inhibits AKT/ERK-dependent signaling and EGFR-dependent proliferation in cells that are resistant to cetuximab and/or panitumumab (Fig. 2and fig. S3).

Activity of anti-EGFR mAbs in spontaneous models of secondary resistance to cetuximab

To mimic the clinical setting, in which EGFR ECD mutations emerge during treatment with anti-EGFR antibodies, we took advantage of preclinical models in which EGFR ECD mutants spontaneously emerged upon exposure to cetuximab (10, 11). We previously showed that resistant cell populations often carry multiple resistance alleles. Accordingly, we first identified cell models that were amenable to single-cell cloning and then obtained individual clones from drug-resistant populations carrying EGFR ECD mutations. Specifically, we studied LIM1215 R5 (G465R), HCA-46 R5 (G465E), and CCK-81 R1 (S464L). As a control, we also included a clone of LIM1215, in which we introduced (knocked in) the EGFR p.S492R variant into the EGFR locus using targeted homologous recombination.

MM-151 effectively inhibited cell proliferation in cells displaying high percentages of mutant EGFR as assessed by droplet digital polymerase chain reaction (ddPCR) (table S3 and figs. S4 to S6). Biochemical analysis revealed suppression of ERK signaling in these cell lines (Fig. 3, A to C).

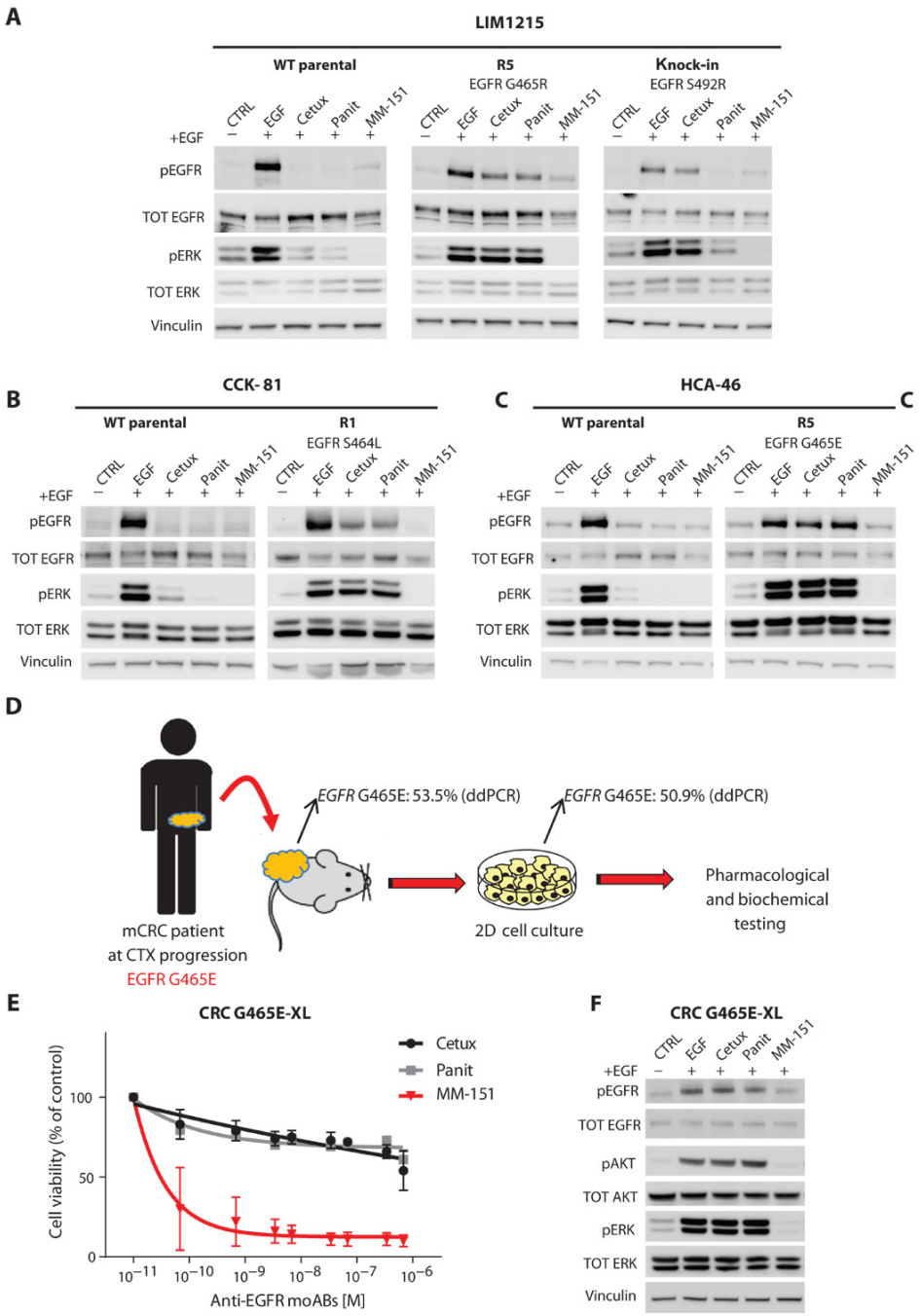

Fig. 3. Effects of MM-151 on cells that acquired EGFR ECD mutations.

(A to C) Effects of cetuximab, panitumumab, and MM-151 on EGFR-dependent signaling in cells carrying EGFR ECD mutations either spontaneously developed (LIM1215 R5, CCK-81 R1, and HCA-46 R5) or knocked-in. The indicated cell models were cultured in the presence of cetuximab, panitumumab, or MM-151 for 2 hours and stimulated with EGF (10 ng/ml) for 15 min. Cell extracts were immunoblotted with the indicated antibodies. Vinculin served as a loading control. (D) Schematic description of the PDX model. The frequency of the G465E mutation in the PDX and in the derivative cell line is indicated. 2D, two-dimensional. (E) Cells derived from a PDX carrying the EGFR p.G465E variant (CRC G465E-XL) were treated for 6 days with increasing concentrations of cetuximab, panitumumab, and MM-151. Cell viability was measured by the ATP assay. The experiment was repeated three times, with mean ± SD plotted at each concentration. The curves were fitted using a nonlinear regression model with a sigmoidal dose response. (F) The same cells were cultured in the presence of cetuximab, panitumumab, and MM-151 for 2 hours and stimulated with EGF (10 ng/ml) for 15 min. Cell extracts were immunoblotted to detect the indicated total or phosphorylated proteins and vinculin (loading control). Cetux, cetuximab; Panit, panitumumab; R1, resistant clone isolated from resistant population 1; R5, resistant clone isolated from resistant population 5.

Activity of EGFR mAbs in patient-derived CRC cells resistant to cetuximab

To further test the efficacy of MM-151 in overcoming secondary resistance to cetuximab, we exploited the CRC xenopatient platform established at our institution (23, 24). We obtained a patient-derived xenograft (PDX) generated from the tumor of a patient who developed secondary resistance to cetuximab and displayed the p.G465E EGFR ECD mutation (Fig. 3D). The CRC cell model, denominated CRC G465E-XL, was successfully established in culture as described in detail in Materials and Methods. ddPCR quantification showed that the G465E mutation is present in a high fraction of the CRC G465E-XL cell population (50.9%), analogous to the one detected in the PDX (53.5%) (Fig. 3D). In a cell proliferation assay, CRC G465E-XL cells were insensitive to both cetuximab and panitumumab, but were sensitive to MM-151 treatment (Fig. 3E), paralleling results previously observed in cell lines expressing EGFR G465E (lentivirus-infected LIM1215 shown in fig. S3 and HCA-46 R5 cells in fig. S6). Analogous to what we had observed in the LIM1215 and HCA-46 cells (Figs. 2 and 3C), biochemical analysis of the CRC G465E-XL cell line showed that MM-151 prominently abrogates ERK signaling in a CRC cell line derived from a patient who developed acquired resistance to cetuximab (Fig. 3F).

Monitoring EGFR ECD mutations in circulating cell-free tumor DNA of patients treated with MM-151

We next sought to investigate the impact of MM-151 in metastatic colorectal patients who developed EGFR ECD mutations as a result of treatment with cetuximab or panitumumab. We used an approach we previously applied to monitor drug resistance mechanisms in circulating cell-free tumor DNA (ctDNA) of CRC patients (25).

The analysis was performed on a subset of serum samples collected on the MM-151 phase 1 study as of February 2015 (NCT01520389) (26). The subset includes 11 CRC patients selected on the basis of availability of serum samples and documented partial response or stable disease on previous anti-EGFR treatment (Table 1).

Table 1. Identification of EGFR ECD mutations in circulating ctDNA of CRC patients.

Serum samples were collected and analyzed by ddPCR in a subset (n = 11) of patients enrolled in the phase 1 clinical trialNCT01520389. These patients represented a subset of study patients who received MM-151 after the emergence of acquired resistance to previous anti-EGFR treatment and had remaining serum available for analysis. The EGFR ECD mutations detected at baseline are noted for each patient. ND, not detected; SD, stable disease; PD, progressive disease. Among the nine patients in this subset who received MM-151 as a monotherapy, two of the three patients who remained on treatment beyond the first scanning interval harbored these EGFR ECD mutations. The remaining patient had indolent disease, having been diagnosed with metastatic disease 7 years earlier and achieving stable disease on five of six previous therapies. One additional patient of the 11 achieved stable disease and received MM-151 in combination with irinotecan.

Subject ID	Study treatment	*EGFR* ECD mutations	Best RECIST v.1.1 response on MM-151
005-30-008	MM-151 monotherapy	ND	PD
010-30-007	MM-151 monotherapy	ND	PD
010-42-020	MM-151 monotherapy	ND	SD
054-62-051	MM-151 monotherapy	*EGFR* p.S464L *EGFR* p.G465R	SD
065-72-065	MM-151 monotherapy	ND	PD
054-92-095	MM-151 monotherapy	*EGFR* p.G465E *EGFR* p.G465R	SD (29.7% reduction)
065-92-103	MM-151 monotherapy	ND	PD
005-02-109	MM-151 monotherapy	ND	PD
065-02-093	MM-151 monotherapy	ND	PD
054-03-106	MM-151 + irinotecan	ND	PD
010-03-086	MM-151 + irinotecan	ND	SD

In ctDNA isolated from 2 of 11 patients (patients 051 and 095), we detected EGFR ECD mutations by ddPCR in the baseline blood draw (before MM-151 treatment) (Table 1). Longitudinal analysis performed in samples collected during the course of MM-151 treatment highlighted that the allelic frequencies of EGFR ECD mutations changed during MM-151 administration (Fig. 4, A and B). Notably, the stark reduction in the allelic frequency of theEGFR p.G465E mutation observed in patient 095 anticipated the marked reduction in tumor volume that was measured about 5 weeks later by computed tomography (CT) scan (Fig. 4A). A reduction and stabilization in EGFR p.S464L and p.G465R mutations, respectively, accompanied the prolonged disease stabilization observed in patient 051 (Fig. 4B). The reversal of the decline in the allelic frequencies of these mutations anticipated the progression by 7 weeks.

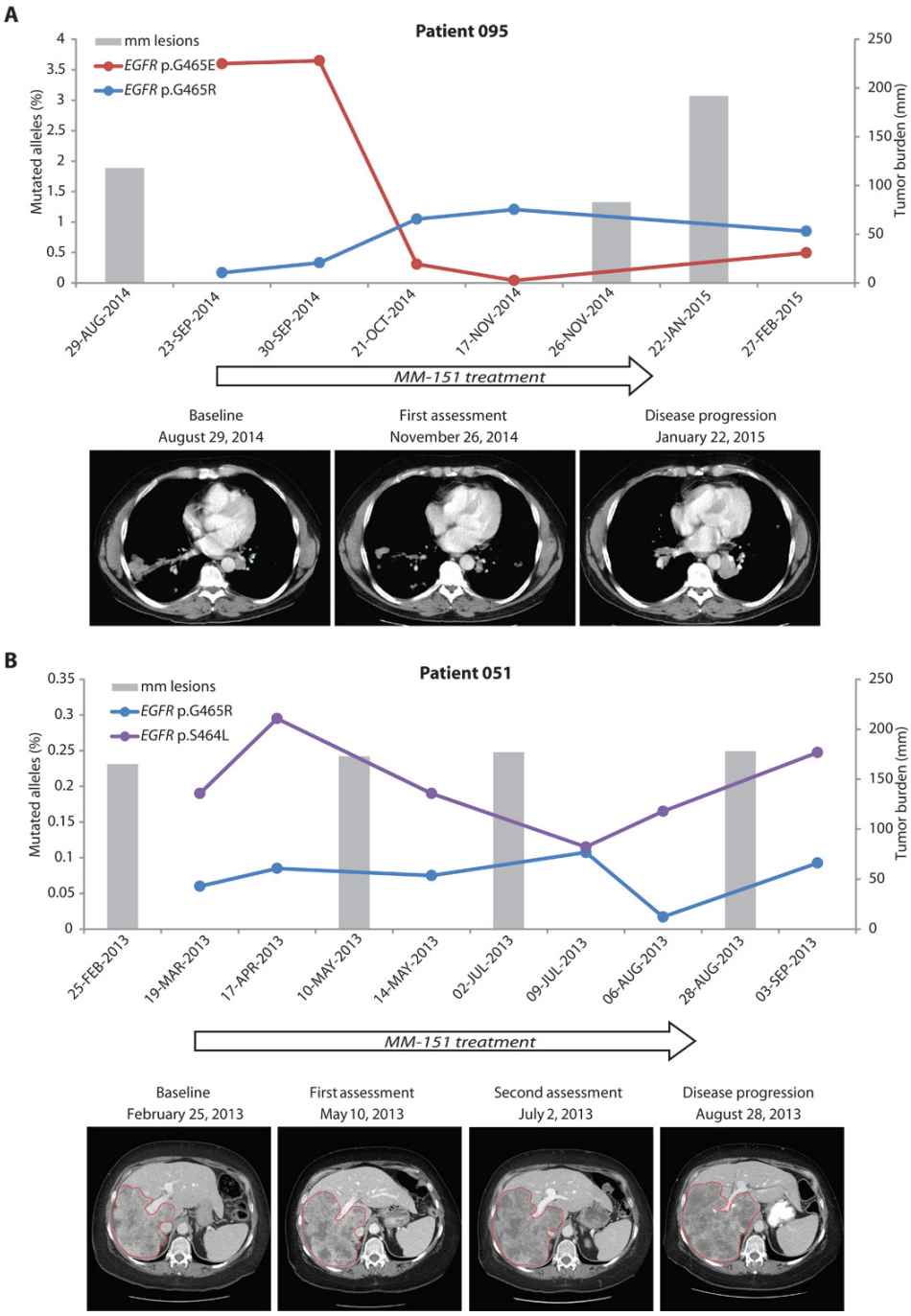

Fig.4.Dynamics of EGFR mutations in the blood of patients treated with MM-151.

(A and B) EGFR ECD mutations were detected by ddPCR in cetuximab-refractory patients 095 (A) and 051 (B) at baseline before MM-151 treatment. The allelic frequency of these mutations declined in ctDNA during MM-151 treatment until progression occurred. CT scans showing the 29% tumor size reduction in patient 095 and disease stabilization in patient 051 during MM-151 treatment. The red outlines in patient 051's scans identify the lesion margins. Tumor burden is measured as the sum of the longest dimension for the target lesions (per RECIST).

DISCUSSION

RTKs play a central role in the pathogenesis of human tumors and are an attractive target for anticancer therapies. However, the onset of drug resistance limits the clinical efficacy of kinase inhibitors. Several studies have shown that acquired resistance to small-molecule kinase inhibitors is accompanied by the emergence of secondary point mutations that affect binding of the drugs to the target protein. Examples include BCR-ABL variants resistant to imatinib (27–29), mutations of ALK and ROS1 insensitive to crizotinib (30–32), and EGFR mutations, which confer resistance to tyrosine kinase inhibitors erlotinib and gefitinib (33, 34). In multiple instances (33, 35), it was found that when secondary mutations arose in the targeted proteins, drug-resistant tumors remained dependent on the oncogenic RTKs for their survival. This finding resulted in the development of compounds capable of inhibiting tyrosine kinases carrying variants resistant to first-generation drugs. For instance, dasatinib and nilotinib have clinical efficacy in imatinib-resistant variants of BCR-ABL (36). Ceritinib is an ALK inhibitor active against mutant variants resistant to the first-generation drug crizotinib (37, 38). The newer EGFR tyrosine kinase inhibitors rociletinib and AZD9291 have shown clinical efficacy in lung cancers with acquired resistance to gefitinib or erlotinib caused by the EGFR p.T790M mutation (39, 40).

Although second-generation and, in some instances, third-generation inhibitors are now available to overcome acquired resistance to small-molecule kinase inhibitors, much less effort has been dedicated to identifying drugs to overcome secondary resistance triggered by the anti-EGFR antibodies cetuximab and panitumumab. A prerequisite for further progress in this area is precise knowledge of the mechanisms of resistance. The discovery that distinct mutations in the ECD of the EGFR occur in about 20% of CRC treated with anti-EGFR antibodies provides opportunities (10, 11, 13) that we exploited in this work. We reasoned that the EGFR variants that emerge upon cetuximab or panitumumab treatment may be targeted by antibodies binding different portions of the receptor. We hypothesized that MM-151 [a drug consisting of a mixture of three EGFR-targeted mAbs (22, 26)] could be effective against cetuximab- and/or panitumumab-resistant tumors that retain dependency on EGFR signaling.

We report that EGF binding activity is maintained in all EGFR ECD mutants and that MM-151 can broadly inhibit this interaction. This indicates that mutant EGFR can be blocked by antibodies targeting epitopes on the receptor that are different from those recognized by cetuximab and/or panitumumab. Pharmacological and biochemical analyses of cell models indicate that MM-151 is active against all known EGFR ECD mutants. The effectiveness of MM-151 is comparable to that of panitumumab against cetuximab-resistant variants that retain sensitivity to panitumumab, namely, EGFR K467T and S492R.

We suggest that colorectal tumors that have become refractory to cetuximab could benefit from second-line EGFR blockade by MM-151. This possibility is supported by two sets of our experiments. First, MM-151 inhibited both cell signaling and proliferation in cells derived from a patient who developed an EGFR ECD mutation upon cetuximab treatment. It is highly possible that clinical treatment with MM-151 could have been beneficial for this subject. The second line of evidence comes from liquid biopsies of 11 patients with available serum samples who relapsed upon previous anti-EGFR treatment and were subsequently treated with MM-151. In 2 of 11 patients in this cohort, we detected EGFR ECD mutations at baseline, a prevalence that is consistent with what we have previously reported in the plasma of patients who relapsed upon anti-EGFR treatment (11). Longitudinal analysis of samples collected over the course of MM-151 treatment shows a decrease or stabilization of EGFR ECD mutant DNA concentrations that paralleled the response assessed by radiological methods.

The two patients presented here are two of the three patients within this selected subgroup that benefited from MM-151 monotherapy treatment. Together, these observations indicate that EGFR ECD variants might be effectively suppressed by in vivo treatment with MM-151. Although our evidence supports the use of new anti-EGFR antibodies to overcome secondary resistance to cetuximab and panitumumab, it also illuminates the potential limitations of clinical trials that exploit a strategy that does not take into account the multiple, potentially concurrent, mechanisms of resistance. Colorectal tumors of patients who develop secondary resistance to EGFR blockade often display heterogeneous mechanisms of resistance, including KRAS and NRAS mutations (6, 11). It is therefore possible that CRC encompassing heterogeneous mechanisms of resistance (for example, RAS and EGFR ECD mutations) would be insensitive to EGFR-

targeted monotherapy, including MM-151. Additional work is needed to further understand the relationship between these concurrent mechanisms of resistance.

In addition to MM-151, other antibodies targeting EGFR are undergoing clinical development in CRC. Among them, Sym004 is a mixture of two synergistic nonoverlapping anti-EGFR antibodies, which has shown encouraging results in early clinical trials (41). Although comparison among new mAbs targeting EGFR is beyond the scope of our work, future studies are warranted to test the ability of Sym004 to bind and inhibit the full complement of EGFR ECD mutations.

In summary, our results suggest that MM-151 may represent a therapeutic opportunity for patients whose tumors develop EGFR ECD mutations as the prevalent mechanism of acquired resistance to cetuximab or panitumumab. Accordingly, MM-151 should undergo prospective clinical evaluation in these patients.

MATERIALS AND METHODS

Study design

This work was initiated on the hypothesis that colorectal tumors displaying EGFR ECD mutations may retain dependence on EGFR beyond progression on previous treatment with the anti-EGFR mAbs cetuximab and panitumumab. This study was designed to assess the ability of the oligoclonal antibody MM-151 to overcome acquired resistance to cetuximab and panitumumab in colorectal tumors harboring mutations in the EGFR ECD.

The study encompasses three main sections. The first involves biochemical and functional assays to evaluate the impact of MM-151 on cells engineered to express ECD mutations. The second section describes the effects of MM-151 on cancer cells that spontaneously developed EGFR ECD mutations and primary cells derived from a patient who acquired an ECD mutation as a result of cetuximab treatment. The final section of the study involves analysis of clinical samples (ctDNA) from mCRC patients who developed acquired resistance to previous anti-EGFR therapy and were then treated with MM-151.

In the first section of the study, we engineered HEK-293 cells to express the seven known EGFRECD variants (p.R451C, p.S464L, p.G465R, p.G465E, p.K467T, p.I491M, and p.S492R) and used the wild type as a control to evaluate the impact of EGFR ECD mutations on ligand-receptor binding. To this end, we exploited the recently developed NanoLuc technology, which detects molecular interactions by measuring BRET from a luminescent protein donor to a fluorescent protein acceptor. We then used the same sensitive technology in the second section of the work to measure the interactions between EGFR ECD mutants and the three anti-EGFR drugs cetuximab, panitumumab, and MM-151. Every NanoLuc binding experiment (both EGF tracer dose response assay and drug displacement assay) was performed at least two times in duplicate. These data were then validated in a second cell line model. In this instance, we expressed the EGFR ECD mutants in a cetuximab-sensitive colorectal cell line, LIM1215, to assess in parallel the effects of cetuximab, panitumumab, and MM-151 on cell proliferation and EGFR-dependent signaling. In vitro drug inhibition assays were performed at least three times in triplicate.

In the second section of the study, we studied colorectal cells that spontaneously developedEGFR ECD mutations during continuous exposure to cetuximab (LIM1215 R5 EGFR-G465R, HCA-46 R5 EGFR-G465E, and CCK-81 R1 EGFR-S464L). To assess the clinical relevance of the findings, we also established a primary culture of cells obtained from a PDX carrying an EGFRECD mutation as a result of progression during cetuximab treatment.

In the last section of the study, we analyzed liquid biopsies from 11 patients who were previously treated with cetuximab or panitumumab and then relapsed. These were samples from a phase 1 study in which MM-151 was administered to patients who relapsed from previous anti-EGFR treatment. ctDNA was extracted at several time points, and EGFR ECD mutants were monitored longitudinally during MM-151 treatment.

Molecular simulation

The EGFR structure was generated in PyMOL software version 1.7.2 using PDB structure 1NQL (www.rcsb.org/pdb/explore.do?structureId=1NQL) (42).

Cellular models

Cell lines resistant to cetuximab and panitumumab have been previously described (11). The HEK-293 and HEK-293T cell lines were purchased from the American Type Culture Collection (ATCC) (CRL-1573 and CRL-3216) (LGC Standards S.r.l.) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS).

Establishment of two-dimensional culture from PDX

A primary CRC cell line was established from tumor tissue obtained from a PDX. Tumor tissue was dissociated into single-cell suspension by mechanical dissociation using the gentleMACS Dissociators (Miltenyi Biotec) and enzymatic degradation of the extracellular matrix using the Tumor Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Cell suspension was collected into 15-ml Falcon tubes and centrifuged at 1200 rpm for 5 min. Supernatants were removed and cell pellets were resuspended with DMEM/F12 medium containing 10% FBS. This process was repeated three times. Then, cell suspensions were filtered into 50-ml Falcon tubes through a 70-µm cell strainer (Falcon). Cells that were not filtered out were resuspended in DMEM/F12 medium containing 10% FBS, gentamicin (50 µg/ml), and 10 µM ROCK inhibitor Y-27632 (Selleck Chemicals Inc.).

Generation of LIM1215 KI EGFR S492R cell line

The LIM1215 parental cell line (43) was obtained from R. Whitehead (Vanderbilt University, Nashville, TN) with permission from the Ludwig Institute for Cancer Research Ltd. (New York, NY). A protocol for generating knock-in cells has already been described (44). The transfer vector for KI EGFR p.S492R mutation (pAAV0223 EGFR p.S492R) was purchased from Horizon Discovery.

DNA constructs, mutagenesis, and lentivirus production

The NanoLuc-EGFR WT vector was purchased from Promega Corp., and the pLX301-EGFR WT construct was a gift from C. Sun and R. Bernards [NKI (Nederlands Kanker Instituut), Amsterdam, Netherlands]. EGFR mutant plasmids containing the seven point mutations (R451C, S464L, G465R, G465E, K467T, I491M, and S492R) were constructed using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies), with the wild-type plasmid as the template DNA. The presence of mutations was confirmed by Sanger sequencing. Lentiviral vector stocks were produced by transient transfection of the p301-EGFR mutated plasmid, the packaging plasmids pMDLg/pRRE and pRSV.REV, and the vesicular stomatitis virus (VSV) envelope plasmid pMD2.VSV-G (45) (12, 5, 2.5, and 3 µg, respectively, for 10-cm dishes) in HEK-293T cells. Viral particles were then purified and concentrated by ultracentrifugation as described (46). Determination of the viral p24 antigen concentration was done by HIV-1 p24 Core Profile ELISA (enzyme-linked immunosorbent assay) (PerkinElmer Life Science Inc.). Cells were transduced in six-well plates (3×10^5 per well in 2 ml of medium) using p24 gag (100 ng/ml) equivalent particles in the presence of polybrene (8 µg/ml) (Sigma).

NanoBRET assay

HEK-293 cells were transiently transfected with FuGENE HD Transfection Reagent (Promega) to allow expression of the EGFR-NanoLuc mutants. In the EGF tracer dose response assay, HEK-293 cells were transfected with plasmids expressing the indicated NanoLuc-EGFR mutants and then treated with increasing doses of EGF tracer (HaloTag-EGF, Promega) in the presence or absence of an excess amount (100 ng/ml) of unlabeled EGF to assess whether EGF tracer can effectively bind to the NanoLuc-EGFR. The NanoBRET Nano-Glo substrate was then added, and the plates were analyzed by the GloMax-Multi Microplate Multimode Reader (Promega). To calculate the raw NanoBRET ratio values, the acceptor emission value (610 nm) was divided by the donor emission value (450 nm) for each sample. Each value was normalized to HaloTag-EGF untreated cells. In the drug displacement assay, transfected HEK cells were treated with increasing doses (from 0 to 10 µg/ml) of cetuximab, panitumumab, and MM-151 and HaloTag-EGF (tracer) at a concentration of 18 ng/ml for 30 min. After NanoBRET measurement, each value was normalized to untreated cells.

Cell viability assays

Cetuximab and panitumumab were obtained from the Pharmacy at Niguarda Hospital (Milan, Italy). MM-151 was obtained from Merrimack Pharmaceuticals. Cell lines were seeded in 100 µl of medium at the following densities: 1.5×10^3 for LIM1215, 2×10^3 for HCA-46, and 3×10^3 for CCK-81 in 96-well culture plates. After serial dilutions, drugs in serum-free medium were added to cells, and medium-only wells were included as controls. Plates were incubated at 37°C in 5% CO_2 for 6 days, after which cell viability was assessed by ATP content using the CellTiter-Glo Luminescent Assay (Promega). Measurements were recorded on a VICTOR X4 plate reader (PerkinElmer). Treated wells were normalized to untreated. Data points represent means ± SD of three independent experiments.

Overview of the clinical trial and collection of serum biomarker samples

A phase 1 study of MM-151 in patients with refractory advanced solid tumors was conducted to evaluate safety and establish a maximum tolerated dose of MM-151 as a monotherapy or in combination with irinotecan (protocol MM-151-01-01-01, NCT# 01520389). As part of this protocol, blood and tumor tissue samples were collected, and written informed consent was obtained from all patients for exploratory biomarker analysis to further characterize and correlate possible biomarkers that may help to predict or evaluate MM-151 response and/or safety. The study was reviewed and approved by the institutional review board at each site [Horizon Oncology Research Inc., Lafayette, IN; South Texas Accelerated Research Therapeutics (START) LLC, San Antonio, TX; Roswell Park Cancer Institute, Buffalo, NY; University of Colorado Denver Anschutz Medical Campus, Aurora, CO], according to local guidelines.

Key eligibility criteria included adult patients (≥18 years of age) with advanced solid tumors refractory to standard treatments, measurable disease per Response Evaluation Criteria in Solid Tumors (RECIST) v.1.1, Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1, and adequate hepatic, renal, and cardiac function.

The study was designed to evaluate escalating doses of MM-151 at various schedules. Patients were treated until progressive disease (according to radiological scans obtained every 8 weeks from the date of the first dose and evaluated on the basis of RECIST v.1.1 criteria), intolerable toxicity, or another reason for discontinuation as assessed by the investigator.

Serum samples were collected at protocol-defined time points to support biomarker analyses. At each collection time point, 9.5 ml of blood was collected in a BD Vacutainer collection tube without additive and allowed to clot for 15 to 30 min at room temperature. The sample was then centrifuged at 3000 rpm for 10 to 15 min to separate cells from serum. The serum was split into two equal aliquots and placed into −80°C storage until shipment to a central storage facility.

Isolation of ctDNA and quantification of genome equivalents (genome equivalent/ml serum)

ctDNA was isolated from serum using the QIAamp Circulating Nucleic Acid Kit (QIAGEN) according to the manufacturer's instructions. ctDNA (3 µl) was then used as template for each quantitative PCR (qPCR) for genome equivalent/ml measurement. All samples were analyzed in triplicate. PCRs were performed in 10-µl final volume containing 5 µl of GoTaq qPCR Master Mix, 2× with CXR Reference Dye (Promega), and LINE-1 (1.5 µmol) forward and reverse primers (LINE-1 qRT-PCR FW-TCACTCAAAGCCGCTCAACTAC; LINE-1 qRT-PCR REV-TCTGCCTTCATTTCGTTATGTACC). DNA at known concentrations was used to build the standard curve.

Droplet digital polymerase chain reaction

Isolated ctDNA was amplified with ddPCR Supermix for Probes (Bio-Rad) using KRAS, NRAS, BRAF, and EGFR assays (PrimePCR ddPCR Mutation Assay, Bio-Rad, and custom-designed; tables S4 and S5). ddPCR was performed according to the manufacturer's protocol (Bio-Rad), and the results were reported as percentage or fractional abundance of mutant DNA alleles relative to total (mutant plus wild type) DNA alleles (25). Eight to 10 µl of DNA template were added to 10 µl of ddPCR Supermix for Probes (Bio-Rad) and 2 µl of the primer/probe 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 min at 95°C, 40 cycles of 94°C for 30 s, 55°C for 1 min followed by 98°C for 10 min (ramp rate 2°C/s). 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 three) were performed for each sample. ddPCR analysis of normal control genomic DNA from cell lines and no DNA template (water) controls was performed in parallel with all samples, including multiple replicates as contamination-free controls.

Immunoblot analysis

Total cellular proteins were extracted by solubilizing the cells in cold EB buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 2 mM EGTA; all reagents were from Sigma-Aldrich, except for Triton X-100 (Fluka)], in the presence of 1 mM sodium orthovanadate, 100 mM sodium fluoride, and a mixture of protease inhibitors [pepstatin, leupeptin, aprotinin (Sigma-Aldrich), and soybean trypsin inhibitor (VWR)]. Extracts were clarified by centrifugation, and protein concentration was determined using BCA Protein Assay Reagent Kit (Thermo Scientific). Western blot detection was performed with enhanced chemiluminescence system (GE Healthcare) and peroxidase-conjugated secondary antibodies (Amersham). The following primary antibodies were used for Western blotting (all from Cell Signaling Technology, except where indicated): anti–phospho-p44/42 ERK (Thr^{202}/Tyr^{204}), anti–p44/42 ERK, anti–phospho-AKT (Ser^{473}), anti-AKT, anti–phospho-EGFR (Tyr^{1068}), anti-EGFR (clone 13G8, Enzo Life Sciences), and anti-vinculin (Sigma-Aldrich). The following day, after 1 hour of incubation with the appropriate secondary antibody, the signal was developed using the ECL system (Amersham Biosciences).

Statistical analysis

All the analyses were performed using the software GraphPad PRISM 6.0. P values were calculated by two-tailed unpaired Student's t test. All values reported in the proliferation assays correspond to means ± SD of at least three independent experiments, each with three experimental replicates. Each NanoBRET assay was performed at least twice with duplicate replicates.

SUPPLEMENTARY MATERIALS

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Fig. S1. Nucleotide sequence of the EGFR p.G465E mutation in HCA-46 R5.

Fig. S2. EGF tracer dose response assay in HEK-293 cells expressing EGFR ECD mutants.

Fig. S3. Effects of EGFR blockade on LIM1215 overexpressing EGFR ectodomain mutants.

Fig. S4. Effects of EGFR blockade on proliferation in LIM1215 cells.

Fig. S5. Effects of EGFR blockade on proliferation in CCK-81 cells.

Fig. S6. Effects of EGFR blockade on proliferation in HCA-46 cells.

Table S1. Mean, SD, and P values for the NanoLuc drug displacement assay.

Table S2. Mean, SD, and P values for the LIM1215 cell viability assay.

Table S3. Effects of anti-EGFR blockade in cetuximab-resistant cells.

Table S4. ddPCR mutation assays for KRAS, BRAF, and NRAS.

Table S5. ddPCR custom-designed assays for EGFR ECD mutations.

REFERENCES AND NOTES

1. A. Bardelli, S. Siena, Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J. Clin. Oncol. 28, 1254–1261 (2010).

2. S. Misale, F. Di Nicolantonio, A. Sartore-Bianchi, S. Siena, A. Bardelli, Resistance to anti-EGFR therapy in colorectal cancer: From heterogeneity to convergent evolution. Cancer Discov. 4, 1269–1280 (2014).

3. S. Misale, R. Yaeger, S. Hobor, E. Scala, M. Janakiraman, D. Liska, E. Valtorta, R. Schiavo, M. Buscarino, G. Siravegna, K. Bencardino, A. Cercek, C.T. Chen, S. Veronese, C. Zanon, A. SartoreBianchi, M.Gambacorta, M. Gallic chio, E. Vakiani, V. Boscaro, E. Medico, M. Weiser, S. Siena, F. Di Nicolantonio, D.Solit, A. Bardelli, Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 486, 532–536 (2012).

4. C. Bettegowda, M. Sausen, R. J. Leary, I. Kinde, Y. Wang, N. Agrawal, B. R. Bartlett, H. Wang, B. Luber, R. M. Alani, E. S. Antonarakis, N. S. Azad, A. Bardelli, H. Brem, J. L. Cameron, C. C. Lee, L. A. Fecher, G. L.Gallia, P. Gibbs, D. Le, R. L. Giuntoli, M. Goggins, M. D. Hogarty, M. Holdhoff, S.-M. Hong, Y. Jiao, H. H.Juhl, J. J. Kim, G. Siravegna, D. A. Laheru, C. Lauricella, M. Lim, E. J. Lipson, S. K. N. Marie, G. J. Netto, K. S. Oliner, A. Olivi, L. Olsson, G. J. Riggins, A. Sartore-Bianchi, K. Schmidt, I.-M. Shih, S. M. Oba-Shinjo, S.Siena, D. Theodorescu, J. Tie, T. T. Harkins, S. Veronese, T.-L. Wang, J. D. Weingart, C. L. Wolfgang, L. D.Wood, D. Xing, R. H. Hruban, J. Wu, P. J. Allen, C. M. Schmidt, M. A. Choti, V. E. Velculescu, K. W. Kinzler,B. Vogelstein, N. Papadopoulos, L. A. Diaz Jr. , Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci. Transl. Med. 6, 224ra24 (2014).

5. L. A. Diaz Jr., R. T. Williams, J. Wu, I. Kinde, J. R. Hecht, J. Berlin, B. Allen, I. Bozic, J. G. Reiter, M. A.Nowak, K. W. Kinzler, K. S. Oliner, B. Vogelstein, The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 486, 537–540 (2012).

6.S. Misale, S. Arena, S. Lamba, G. Siravegna, A. Lallo, S. Hobor, M. Russo, M. Buscarino, L. Lazzari, A.Sartore-Bianchi, K. Bencardino, A. Amatu, C. Lauricella, E. Valtorta, S. Siena, F. Di Nicolantonio, A.Bardelli, Blockade of EGFR

and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer. Sci. Transl. Med. 6, 224ra26 (2014).

7. K. Yonesaka, K. Zejnullahu, I. Okamoto, T. Satoh, F. Cappuzzo, J. Souglakos, D. Ercan, A. Rogers, M.Roncalli, M. Takeda, Y. Fujisaka, J. Philips, T. Shimizu, O. Maenishi, Y. Cho, J. Sun, A. Destro, K. Taira, K.Takeda, T. Okabe, J. Swanson, H. Itoh, M. Takada, E. Lifshits, K. Okuno, J. A. Engelman, R. A. Shivdasani,K. Nishio, M. Fukuoka, M. Varella-Garcia, K. Nakagawa, P. A. Jänne, Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci. Transl. Med. 3, 99ra86 (2011).

8. A. Bardelli, S. Corso, A. Bertotti, S. Hobor, E. Valtorta, G. Siravegna, A. Sartore-Bianchi, E. Scala, A.Cassingena, D. Zecchin, M. Apicella, G. Migliardi, F. Galimi, C. Lauricella, C. Zanon, T. Perera, S. Veronese, G. Corti, A. Amatu, M. Gambacorta, L. A. Diaz Jr., M. Sausen, V. E. Velculescu, P. Comoglio, L. Trusolino, F.Di Nicolantonio, S. Giordano, S. Siena, Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer Discov. 3, 658–673 (2013).

9. S. Mohan, E. Heitzer, P. Ulz, I. Lafer, S. Lax, M. Auer, M. Pichler, A. Gerger, F. Eisner, G. Hoefler, T.Bauernhofer, J. B. Geigl, M. R. Speicher, Changes in colorectal carcinoma genomes under anti-EGFR therapy identified by whole-genome plasma DNA sequencing. PLOS Genet. 10, e1004271 (2014).

10. C. Montagut, A. Dalmases, B. Bellosillo, M. Crespo, S. Pairet, M. Iglesias, M. Salido, M. Gallen, S.Marsters, S. P. Tsai, A. Minoche, S. Seshagiri, S. Serrano, H. Himmelbauer, J. Bellmunt, A. Rovira, J.Settleman, F. Bosch, J. Albanell, Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. Nat. Med. 18, 221–223(2012).

11. S. Arena, B. Bellosillo, G. Siravegna, A. Martínez, I. Cañadas, L. Lazzari, N. Ferruz, M. Russo, S. Misale, I. González, M. Iglesias, E. Gavilan, G. Corti, S. Hobor, G. Crisafulli, M. Salido, J. Sánchez, A. Dalmases, J.Bellmunt, G. De Fabritiis, A. Rovira, F. Di Nicolantonio, J. Albanell, A. Bardelli, C. Montagut, Emergence of multiple EGFR extracellular mutations during cetuximab treatment in colorectal cancer. Clin. Cancer Res. 21, 2157–2166 (2015).

12. M. P. Morelli, M. J. Overman, A. Dasari, S. M. A. Kazmi, T. Mazard, E. Vilar, V. K. Morris, M. S. Lee, D.Herron, C. Eng, J. Morris, B. K. Kee, F. Janku, F. L. Deaton, C. Garrett, D. Maru, F. Diehl, P. Angenendt, S.Kopetz, Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. Ann. Oncol. 26, 731–736 (2015).

13. K. Newhall, T. Price, M. Peeters, T. W. Kim, J. Li, S. Cascinu, P. Ruff, A. S. Suresh, A. Thomas, S.Tjulandin, S. Ogbagabriel, M. Boedigheimer, S. Sexson, K. Zhang, S. Murugappan, R. Sidhu, Frequency of S492R mutations in the epidermal growth factor receptor: Analysis of plasma DNA from metastatic colorectal cancer patients treated with panitumumab or cetuximab monotherapy; ESMO 16th World Congress on Gastrointestinal Cancer 2014. Ann. Oncol. 25 (Suppl. 2), ii109 (2014).

14. L. Gianni, T. Pienkowski, Y.-H. Im, L. Roman, L.M. Tseng, M.C. Liu, A. Lluch, E. Staroslawska, J. de la Haba-Rodriguez, S.-A. Im, J. L. Pedrini, B. Poirier, P. Morandi, V. Semiglazov, V. Srimuninnimit, G. Bianchi,T. Szado, J. Ratnayake, G. Ross, P. Valagussa, Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): A randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 13, 25–32 (2012).

15. G. M. Blumenthal, N. S. Scher, P. Cortazar, S. Chattopadhyay, S. Tang, P. Song, Q. Liu, K. Ringgold, A. M.Pilaro, A. Tilley, K. E. King, L. Graham, B. L. Rellahan, W. C. Weinberg, B. Chi, C. Thomas, P. Hughes, A.Ibrahim, R. Justice, R. Pazdur, First FDA approval of dual anti-HER2 regimen: Pertuzumab in combination with trastuzumab and docetaxel for HER2-positive metastatic breast cancer. Clin. Cancer Res. 19,4911–4916 (2013).

17. S. M. Swain, J. Baselga, S.-B. Kim, J. Ro, V. Semiglazov, M. Campone, E. Ciruelos, J.-M. Ferrero, A.Schneeweiss, S. Heeson, E. Clark, G. Ross, M. C. Benyunes, J. Cortés, Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N. Engl. J. Med. 372, 724–734 (2015).

18. L. M. Friedman, A. Rinon, B. Schechter, L. Lyass, S. Lavi, S. S. Bacus, M. Sela, Y. Yarden, Synergistic down-regulation of receptor tyrosine kinases by combinations of mAbs: Implications for cancer immunotherapy. Proc. Natl. Acad. Sci. U.S.A. 102, 1915–1920 (2005).

19. V. Kamat, J. M. Donaldson, C. Kari, M. R. D. Quadros, P. I. Lelkes, I. Chaiken, S. Cocklin, J. C. Williams, E.Papazoglou, U. Rodeck, Enhanced EGFR inhibition and distinct epitope recognition by EGFR antagonistic MABS C225 and 425. Cancer Biol. Ther. 7, 726–733 (2008).

20. T. Ben-Kasus, B. Schechter, S. Lavi, Y. Yarden, M. Sela, Persistent elimination of ErbB-2/HER2-overexpressing tumors using combinations of monoclonal antibodies: Relevance of receptor endocytosis. Proc. Natl. Acad. Sci. U.S.A. 106, 3294–3299 (2009).

21. R. M. Perera, Y. Narita, F. B. Furnari, H. K. Gan, C. Murone, M. Ahlkvist, R. B. Luwor, A. W. Burgess, E.Stockert, A. A. Jungbluth, L. J. Old, W. K. Cavenee, A. M. Scott, T. G. Johns, Treatment of human tumor xenografts with monoclonal antibody 806 in combination with a prototypical epidermal growth factor receptor–specific antibody generates enhanced antitumor activity. Clin. Cancer Res. 11, 6390–6399(2005).

22. C. I. Spiridon, M.-A. Ghetie, J. Uhr, R. Marches, J.-L. Li, G.-L. Shen, E. S. Vitetta, Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. Clin. Cancer Res. 8, 1720–1730 (2002).

23. J. D. Kearns, R. Bukhalid, M. Sevecka, G. Tan, N. Gerami-Moayed, S. L. Werner, N. Kohli, O. Burenkova, C. M. Sloss, A. M. King, J. B. Fitzgerald, U. B. Nielsen, B. B. Wolf, Enhanced targeting of the EGFR network with MM-151, an oligoclonal anti-EGFR antibody therapeutic. Mol. Cancer Ther. 14, 1625–1636 (2015).

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

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

26. G. Siravegna, B. Mussolin, M. Buscarino, G. Corti, A. Cassingena, G. Crisafulli, A. Ponzetti, C.Cremolini, A. Amatu, C. Lauricella, S. Lamba, S. Hobor, A. Avallone, E. Valtorta, G. Rospo, E. Medico, V.Motta, C. Antoniotti, F. Tatangelo,

B. Bellosillo, S. Veronese, A. Budillon, C. Montagut, P. Racca, S.Marsoni, A. Falcone, R.B. Corcoran, F. Di Nicolantonio, F. Loupakis, S. Siena, A. Sartore-Bianchi, A.Bardelli, Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat. Med. 21, 827 (2015).

27. C. H. Lieu, M. Beeram, W. Harb, J. Kearns, C. M. Sloss, R. Nering, A. Adjei, B. B. Wolf, Safety, pharmacology, and preliminary clinical activity of MM-151: An oligoclonal anti-EGFR therapeutic in patients with cetuximab-resistant CRC and other refractory solid tumors. J. Clin. Oncol. 33 (Suppl. 3),abstract 647 (2015).

28. J. S. Khorashad, T. W. Kelley, P. Szankasi, C. C. Mason, S. Soverini, L. T. Adrian, C. A. Eide, M. S.Zabriskie, T. Lange, J. C. Estrada, A. D. Pomicter, A. M. Eiring, I. L. Kraft, D. J. Anderson, Z. Gu, M. Alikian,A. G. Reid, L. Foroni, D. Marin, B. J. Druker, T. O'Hare, M. W. Deininger, BCR-ABL1 compound mutations in tyrosine kinase inhibitor–resistant CML: Frequency and clonal relationships. Blood 121, 489–498 (2013).

29. S. Soverini, C. De Benedittis, K. Machova Polakova, A. Brouckova, D. Horner, M. Iacono, F. Castagnetti, G.Gugliotta, F. Palandri, C. Papayannidis, I. Iacobucci, C. Venturi, M. T. Bochicchio, H. Klamova, F. Cattina, D.Russo, P. Bresciani, G. Binotto, B. Giannini, A. Kohlmann, T. Haferlach, A. Roller, G. Rosti, M. Cavo, M.Baccarani, G. Martinelli, Unraveling the complexity of tyrosine kinase inhibitor–resistant populations by ultra-deep sequencing of the BCR-ABL kinase domain. Blood 122, 1634–1648 (2013).

30. D. L. Gibbons, S. Pricl, P. Posocco, E. Laurini, M. Fermeglia, H. Sun, M. Talpaz, N. Donato, A. Quintás-Cardama , Molecular dynamics reveal BCR-ABL1 polymutants as a unique mechanism of resistance to PAN-BCR-ABL1 kinase inhibitor therapy. Proc. Natl. Acad. Sci. U.S.A. 111, 3550–3555 (2014).

31. Y. L. Choi, M. Soda, Y. Yamashita, T. Ueno, J. Takashima, T. Nakajima, Y. Yatabe, K. Takeuchi, T.Hamada, H. Haruta, Y. Ishikawa, H. Kimura, T. Mitsudomi, Y. Tanio, H. Mano, EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. N. Engl. J. Med. 363, 1734–1739 (2010).

32. R. Katayama, A. T. Shaw, T. M. Khan, M. Mino-Kenudson, B. J. Solomon, B. Halmos, N. A. Jessop, J. C.Wain, A. T. Yeo, C. Benes, L. Drew, J. C. Saeh, K. Crosby, L. V. Sequist, A. J. Iafrate, J. A. Engelman, Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. Sci. Transl. Med. 4,120ra17 (2012).

33. M. M. Awad, R. Katayama, M. McTigue, W. Liu, Y.-L. Deng, A. Brooun, L. Friboulet, D. Huang, M. D. Falk,S. Timofeevski, K. D. Wilner, E. L. Lockerman, T. M. Khan, S. Mahmood, J. F. Gainor, S. R. Digumarthy, J. R. Stone, M. Mino-Kenudson, J. G. Christensen, A. J. Iafrate, J. A. Engelman, A. T. Shaw, Acquired resistance to crizotinib from a mutation in CD74–ROS1. N. Engl. J. Med. 368, 2395–2401 (2013).

34. W. Pao, V. A. Miller, K. A. Politi, G. J. Riely, R. Somwar, M. F. Zakowski, M. G. Kris, H. Varmus, Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLOS Med. 2, e73 (2005).

35. L. V. Sequist, B. A. Waltman, D. Dias-Santagata, S. Digumarthy, A. B. Turke, P. Fidias, K. Bergethon, A. T. Shaw, S. Gettinger, A. K. Cosper, S. Akhavanfard, R. S. Heist, J. Temel, J. G. Christensen, J. C. Wain, T. J.Lynch, K. Vernovsky, E. J. Mark, M. Lanuti, A. J. Iafrate, M. Mino-Kenudson, J. A. Engelman, Genotypic and

36. F. Heidel, F. K. Solem, F. Breitenbuecher, D. B. Lipka, S. Kasper, M. H. Thiede, C. Brandts, H. Serve, J.Roesel, F. Giles, E. Feldman, G. Ehninger, G. J. Schiller, S. Nimer, R. M. Stone, Y. Wang, T. Kindler, P. S.Cohen, C. Huber, T. Fischer, Clinical resistance to the kinase inhibitor PKC412 in acute myeloid leukemia by mutation of Asn-676 in the FLT3 tyrosine kinase domain. Blood 107, 293–300 (2006).

37. A. Quintás-Cardama, H. Kantarjian, J. Cortes, Imatinib and beyond—Exploring the full potential of targeted therapy for CML. Nat. Rev. Clin. Oncol. 6, 535–543 (2009).

38. A. T. Shaw, D.-W. Kim, R. Mehra, D. S. W. Tan, E. Felip, L. Q. M. Chow, D. R. Camidge, J. Vansteenkiste, S. Sharma, T. De Pas, G. J. Riely, B. J. Solomon, J. Wolf, M. Thomas, M. Schuler, G. Liu, A. Santoro, Y. Y.Lau, M. Goldwasser, A. L. Boral, J. A. Engelman, Ceritinib in ALK-rearranged non–small-cell lung cancer.N. Engl. J. Med. 370, 1189–1197 (2014).

39. L. Friboulet, N. Li, R. Katayama, C. C. Lee, J. F. Gainor, A. S. Crystal, P.-Y. Michellys, M. M. Awad, N.Yanagitani, S. Kim, A. C. Pferdekamper, J. Li, S. Kasibhatla, F. Sun, X. Sun, S. Hua, P. McNamara, S.Mahmood, E. L. Lockerman, N. Fujita, M. Nishio, J. L. Harris, A. T. Shaw, J. A. Engelman, The ALK inhibitor ceritinib overcomes crizotinib resistance in non–small cell lung cancer. Cancer Discov. 4, 662–673(2014).

40. P. A. Jänne, J. C.-H. Yang, D.-W. Kim, D. Planchard, Y. Ohe, S. S. Ramalingam, M.-J. Ahn, S.-W. Kim, W.-C. Su, L. Horn, D. Haggstrom, E. Felip, J.-H. Kim, P. Frewer, M. Cantarini, K. H. Brown, P. A. Dickinson, S.Ghiorghiu, M. Ranson, AZD9291 in EGFR inhibitor–resistant non–small-cell lung cancer. N. Engl. J. Med.372, 1689–1699 (2015).

41. L. V. Sequist, J.-C. Soria, J. W. Goldman, H. A. Wakelee, S. M. Gadgeel, A. Varga, V.Papadimitrakopoulou, B. J. Solomon, G. R. Oxnard, R. Dziadziuszko, D. L. Aisner, R. C. Doebele, C.Galasso, E. B. Garon, R. S. Heist, J. Logan, J. W. Neal, M. A. Mendenhall, S. Nichols, Z. Piotrowska, A. J.Wozniak, M. Raponi, C. A. Karlovich, S. Jaw-Tsai, J. Isaacson, D. Despain, S. L. Matheny, L. Rolfe, A. R.Allen, D. R. Camidge, Rociletinib in EGFR-mutated non–small-cell lung cancer. N. Engl. J. Med. 372,1700–1709 (2015).

42. R. Dienstmann, A. Patnaik, R. Garcia-Carbonero, A. Cervantes, M. Benavent, S. Roselló, B. B. J. Tops,R. S. van der Post, G. Argilés, N. J. Ø. Skartved, U. H. Hansen, R. Hald, M. W. Pedersen, M. Kragh, I. D.Horak, S. Braun, E. Van Cutsem, A. W. Tolcher, J. Tabernero, Safety and activity of the first-in-class Sym004 anti-EGFR antibody mixture in patients with refractory colorectal cancer. Cancer Discov. 5,598–609 (2015).

43. K. M. Ferguson, M. B. Berger, J. M. Mendrola, H.-S. Cho, D. J. Leahy, M. A. Lemmon, EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol. Cell 11, 507–517(2003).

44. R. H. Whitehead, F. A. Macrae, D. J. B. St. John, J. Ma, A colon cancer cell line (LIM1215) derived from a patient with inherited nonpolyposis colorectal cancer. J. Natl. Cancer Inst. 74, 759–765 (1985).

45. F. Di Nicolantonio, S. Arena, M. Gallicchio, D. Zecchin, M. Martini, S. E. Flonta, G. M. Stella, S. Lamba, C.Cancelliere, M. Russo, M. Geuna, G. Appendino, R. Fantozzi, E. Medico, A. Bardelli, Replacement of normal with mutant alleles in the genome of normal human cells unveils mutation-specific drug responses. Proc. Natl. Acad. Sci. U.S.A. 105, 20864–20869 (2008).

46. T. Dull, R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono, L. Naldini, A third-generation lentivirus vector with a conditional packaging system. J. Virol. 72, 8463–8471 (1998).

47. A. Follenzi, L. Naldini, HIV-based vectors. Preparation and use. Methods Mol. Med. 69, 259–274(2002).

48. S. Li, K. R. Schmitz, P. D. Jeffrey, J. J. W. Wiltzius, P. Kussie, K. M. Ferguson, Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 7, 301–311 (2005).

49. M. Voigt, F. Braig, M. Göthel, A. Schulte, K. Lamszus, C. Bokemeyer, M. Binder, Functional dissection of the epidermal growth factor receptor epitopes targeted by panitumumab and cetuximab. Neoplasia14, 1023–1031 (2012).

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