**Title: MM-151 overcomes acquired resistance to cetuximab and panitumumab
in colorectal cancers harboring EGFR extracellular domain mutations**

Authors: Sabrina Arena^{1,2}, Giulia Siravegna^{1,3}, Benedetta Mussolin¹, Jeffrey D. Kearns⁴, Beni B. Wolf⁴, Sandra Misale^{1,†}, Luca Lazzari^{1,3}, Andrea Bertotti^{1,3}, Livio Trusolino^{1,3}, Alex A. Adjei⁵, Clara Montagut^{6,7}, Federica Di Nicolantonio^{1,3}, Rachel Nering⁴ and Alberto Bardelli^{1,3,*}

Affiliations:

¹ Candiolo Cancer Institute – FPO, IRCCS, Candiolo, Torino, Italy

² FIRC Institute of Molecular Oncology (IFOM), Milano, Italy

³ University of Torino, Department of Oncology, Candiolo, Torino, Italy

⁴ Merrimack Pharmaceuticals, Cambridge, MA, USA

⁵ Roswell Park Cancer Institute, Buffalo, NY, USA

⁶ Medical Oncology Department, Hospital del Mar, Barcelona, Spain

⁷ Cancer Research Program, FIMIM, Hospital del Mar, Barcelona, Spain

[†]present address: Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA 02129, USA

*To whom correspondence should be addressed: alberto.bardelli@unito.it

One Sentence Summary: The oligoclonal antibody MM-151 effectively targets colorectal cancers which acquired EGFR extracellular domain mutations as a results of previous treatment with cetuximab and panitumumab.

Abstract: The anti-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 extracellular domain. 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 clinical use of MM-151 in patients who become resistant to cetuximab or panitumumab as a result of EGFR ECD mutations.

Introduction

The anti-EGFR monoclonal antibodies (moAbs) cetuximab and panitumumab are effective in a subset of RAS/BRAF wild-type metastatic colorectal cancers (mCRC). 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, BRAF)(3-6) or activation of parallel receptor tyrosine kinase pathways (HER2 and MET)(7-9) can confer acquired resistance to cetuximab or panitumumab. We and others further reported that mutations in the *EGFR* extracellular domain (ECD) (p.S492R, p.R451C, p.S464L, p.G465R, p.K467T, p.I491M) also mediate secondary resistance to EGFR blockade in colorectal cancer(10-13). Notably, the majority 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 prior treatment with anti-EGFR moAbs. 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 binds to different epitopes located in the EGFR extracellular domain. The use of mixtures of monoclonal antibodies recognizing distinct epitopes of receptor tyrosine kinases 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 moAb combinations targeting EGFR on multiple, non-overlapping epitopes. Among these, MM-151 is a third-generation EGFR inhibitor consisting of three fully-human IgG1s that simultaneously engage distinct, non-overlapping epitopes on EGFR(22). MM-151 has demonstrated superiority to currently approved and investigational moABs in preclinical models, displaying improvements in EGFR pathway inhibition and downstream signaling, as well as enhanced down-regulation of the EGF receptor and engagement of innate immune responses(22). Notably, MM-151 targets regions of the EGFR distinct from those affected by ECD mutations (Fig. 1A). In this work, we tested the hypothesis that CRCs that develop resistance to cetuximab or panitumumab through EGFR ECD mutations might be sensitive to EGFR blockade by MM-151.

Results

Impact of EGFR extracellular domain mutations on ligand-receptor binding

We previously identified six distinct *EGFR* ECD mutations in colorectal cancer cells and patients that 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 EGFR receptors can interact with the ligand, 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, p.S492R) were expressed in 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 p.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 EGF receptors 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 extracellular domain 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 WT 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 monoclonal antibodies, 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.

We found that p.R451C expressing cells were still sensitive to cetuximab treatment. 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/AKT signaling pathway. 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.

Activity of anti-EGFR moAbs 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 PCR (Table S3 and Fig. S4-6). Biochemical analysis revealed significant suppression of ERK signaling in these cell lines (Fig. 3A-C).

Activity of EGFR moAbs 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). 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* extracellular domain mutation (Fig. 3D). The CRC cell model, denominated CRC G465E-XL, was successfully established in culture as described in detail in the 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 p.G465E (HCA-46 R5 cells and lentivirus-infected LIM1215). Analogous to what we had observed in the LIM1215 cells, 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 that 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(24).

The analysis was performed on a subset of serum samples collected on the MM-151 phase 1 study as of December 2014 (NCT01520389)(25). The subset includes 11 CRC patients selected based upon availability of serum samples and documented partial response (PR) or stable disease (SD) on prior anti-EGFR treatment (Table 1).

In ctDNA isolated from 2 out 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. 4A and C). Notably, the stark reduction in the allelic frequency of the *EGFR* p.G465E mutation observed in patient 095 anticipated the significant reduction in tumor volume that was measured approximately four weeks later by CT scan (Fig. 4A and B). A reduction and stabilization in *EGFR* p.S464L and p.G465R mutations, respectively, accompanied the prolonged disease stabilization observed in patient 051 (Fig. 4C and D). The

reversal of the decline in the allelic frequencies of these mutations anticipated the progression at 24 weeks.

Discussion

Receptor tyrosine kinases (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(26-28), mutations of ALK and ROS1 insensitive to crizotinib(29-31), and EGFR mutations, which confer resistance to tyrosine kinase inhibitors erlotinib and gefitinib(32, 33). In multiple instances, it was found that when secondary mutations arose in the targeted proteins, drug-resistant tumors remained dependent upon 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(34). Ceritinib is an ALK inhibitor active against mutant variants resistant to the first-generation drug crizotinib(35, 36). 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(37, 38).

Although second 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 extracellular domain of the EGF receptor occur in approximately 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 moAbs(22, 25)] 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 newly discovered *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 which 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. Firstly, 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 who relapsed upon previous anti-EGFR treatment and

were subsequently treated with MM-151. In 2/11 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 of EGFR ECD mutant DNA concentrations that paralleled the response assessed by radiological methods. Altogether, 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 which 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 MM151, other antibodies targeting EGFR are undergoing clinical development in CRC. Among them, Sym004 is a mixture of two synergistic non-overlapping anti-EGFR antibodies, which has shown encouraging results in early clinical trials(39). Although comparison amongst new moAbs 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 suggests 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, MM151 should undergo prospective clinical evaluation in this setting.

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 prior treatment with the anti-EGFR moAbs 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 extracellular domain (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 EGFR ECD variants (p.R451C, p.S464L, p.G465R, p.G465E, p.K467T, p.I491M, p.S492R) and used the WT as a control to evaluate the impact of EGFR extracellular domain 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 developed EGFR 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 PDXcarrying an EGFR ECD 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 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 (<http://www.rcsb.org/pdb/explore.do?structureId=1NQL>).

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 ATCC (CRL-1573 and CRL-3216) (LGC Standards S.r.l) and cultured in DMEM (Invitrogen) supplemented with 10% FBS.

Establishment of 2D culture from patient-derived xenograft

A primary colorectal cancer cell line was established from tumor tissue obtained from a patient-derived xenograft. 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 minutes. Supernatants were removed and cell pellets were resuspended with DMEM/F12 medium containing 10% FBS. This process was repeated 3 times. Then, cell suspensions were filtered into 50 ml Falcon tubes through a 70 μ m cell strainer

(Falcon). Cells that were not filtered out were resuspended in DMEM-F12 medium containing 10% FBS, gentamicin, and 10 μ M ROCK inhibitor Y-27632 (Selleck Chemicals Inc).

Generation of LIM1215 KI EGFR S492R cell line

The LIM1215 parental cell line⁽⁴⁰⁾ was obtained from Prof. Robert Whitehead, Vanderbilt University, Nashville, with permission from the Ludwig Institute for Cancer Research Ltd, New York, NY. A protocol for generating knock-in cells has already been described⁽⁴¹⁾. The transfer vector for KI *EGFR* p.S492R mutation was purchased from Horizon Discovery (pAAV0223 *EGFR* p.S492R, Horizon Discovery).

DNA constructs, mutagenesis and lentivirus production

The nanoLuc-EGFR WT vector was purchased from Promega Corporation, and the pLX301-EGFR WT construct was a generous gift from Dr. C. Sun and Prof R. Bernards (NKI, Amsterdam, the 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 WT 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 (12, 5, 2.5, and 3 μ g, respectively, for 10-cm dishes) in HEK-293T cells. Viral particles were then purified and concentrated by ultracentrifugation as described⁽⁴²⁾. Determination of the viral p24 antigen concentration was done by

HIV-1 p24 Core profile ELISA (Perkin-Elmer Life Science Life Science, Inc.). Cells were transduced in 6-well plates (3×10^5 per well in 2 mL medium) using 100 ng/mL of p24 gag equivalent particles in the presence of 8 µg/ml polybrene (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 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 minutes. After NanoBRET measurement, each value was normalized to untreated cells.

Cell viability assays

Cetuximab and panitumumab were obtained from the Pharmacy at Niguarda Ca' Granda Hospital, Milan, Italy. MM-151 was obtained from Merrimack Pharmaceuticals. Cell lines were seeded in 100 µL 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% CO₂ for 6 days, after which cell viability was assessed by ATP content using the Cell Titer-Glo Luminescent Assay (Promega). Measurements were recorded on a Victor-X4 plate reader (PerkinElmer). Treated wells were normalized to untreated. Data points represent mean $\pm$ 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, according to local guidelines.

Key eligibility criteria included adult patients 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 (assessed by radiological scans every eight weeks from the date of first dose), 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 blood was collected in a red top tube without additive and allowed to clot for 15-30 minutes at room temperature. The sample was then centrifuged at 3000 rpm for 10-15 minutes 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 (GE/ml serum)

ctDNA was isolated from serum using the QIAamp Circulating Nucleic Acid Kit (QIAGEN) according to the manufacturer's instructions. 6 μL of ctDNA were then used as template for each qPCR reaction for GE/ml measurement. All samples were analyzed in triplicate. PCR reactions were performed in 10 μL final volume containing 5 μL GoTaq qPCR Master Mix, 2X with CXR Reference Dye (Promega) and LINE-1 [1.5 μmol] forward and reverse primers. DNA at known concentrations was used to build the standard curve. Primer sequences are available upon request.

Droplet digital PCR

Isolated gDNA 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). Droplet digital PCR (ddPCR) was performed according to

the manufacturer's protocol and the results reported as percentage or fractional abundance of mutant DNA alleles relative to total (mutant plus wild type) DNA alleles. Eight to 10 μ l of DNA template was added to 10 μ l of ddPCR Supermix for Probes (Bio-Rad) and 2 μ l of the primer/probe mixture. This 20 μ l sample was added to 70 μ l 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 three) 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 samples, including multiple replicates as contamination-free controls. Probes and primer sequences are available upon request.

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 from Fluka) in the presence of 1 mM sodium orthovanadate, 100 mM sodium fluoride, and a mixture of protease inhibitors (pepstatin, leupeptin, aprotinin, and STI). Extracts were clarified by centrifugation, and protein concentration was determined using

BCA protein assay reagent kit (Thermo). 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 (Thr202/Tyr204); anti-p44/42 ERK; anti-phospho-AKT (Ser473), anti-AKT; anti-phospho EGFR (Tyr1068); anti-EGFR (clone13G8, Enzo Life Sciences); 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 unpaired Student's t-test. All values reported in the proliferation assays correspond to means $\pm$ 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

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 WT parental and cetuximab resistant cells expressing *EGFR* ectodomain mutations.

Fig. S5. Effects of EGFR blockade on proliferation in CCK-81 wt parental and cetuximab resistant cells expressing *EGFR* p.S464L mutation.

Fig. S6. Effects of EGFR blockade on proliferation in HCA-46 parental and cetuximab resistant cells expressing *EGFR* p.G465E mutation.

Table S1. Mean, standard deviation and p values for the Nanoluc Drug displacement assay (Fig.1B).

Table S2. Mean, standard deviation and p values for the LIM1215 cell viability assay (Fig. 1C).

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

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. Sartore-Bianchi, M. Gambacorta, M. Gallicchio, 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. Lubber, 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. Marie, G. J. Netto, K. S. Oliner, A. Olivi, L. Olsson, G. J. Riggins, A. Sartore-Bianchi, K. Schmidt, M. Shih I, 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**, 224ra224 (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**, 224ra226 (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, 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. Somasekar, 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. 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. Kim, S. Cascinu, P. Ruff, A. Suresh, A. Thomas, S. Ogbagabriel, M. Boedigheimer, S. Sexson, K. Zhang, S. Murugappan, 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. *Annals of Oncology* **25 (suppl_2): ii105-ii117**. [10.1093/annonc/mdu193](https://doi.org/10.1093/annonc/mdu193) (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. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 4911-4916 (2013).
 16. 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. Cortes, Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *The New England journal of medicine* **372**, 724-734 (2015).
 17. 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).
 18. V. Kamat, J. M. Donaldson, C. Kari, M. R. 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).
 19. 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).
20. R. M. Perera, Y. Narita, F. B. Furnari, H. K. Gan, C. Murone, M. Ahlqvist, 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).
 21. 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).
 22. 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*, (2015).
 23. G. M. Andrea Bertotti, Francesco Galimi, Francesco Sassi, Davide Torti,, D. C. Claudio Isella, Federica Di Nicolantonio, Michela Buscarino, Dario Ribero,, A. M. Nadia Russolillo, Paolo Massucco, Alberto Pisacane, Luca Molinaro,, A. S.-B. Emanuele Valtorta, Mauro Risio, Lorenzo Capussotti,, S. S. Marcello Gambacorta, Enzo Medico, Anna Sapino, Silvia Marsoni,, A. B. a. L. T. Paolo M. Comoglio, A molecularly annotated platform of patient-derived xenografts ('xenopatients') identifies HER2 as an effective

- therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discovery*, (2011).
24. 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*, (2015).
 25. 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; abstr 647)**, (2015).
 26. 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).
 27. 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).
28. D. L. Gibbons, S. Prichard, P. Posocco, E. Laurini, M. Fermeiglia, H. Sun, M. Talpaz, N. Donato, A. Quintas-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).
29. 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).
30. 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**, 120ra117 (2012).
31. 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).
32. 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).

33. 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 Histological Evolution of Lung Cancers Acquiring Resistance to EGFR Inhibitors. *Sci Transl Med* **3**, 75ra26 (2011).
34. 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).
35. A. T. Shaw, D. W. Kim, R. Mehra, D. S. Tan, E. Felip, L. Q. 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. Borla, J. A. Engelman, Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med* **370**, 1189-1197 (2014).
36. 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. Pflerdekamper, 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).
37. P. A. Janne, J. C. 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).
38. 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).
 39. R. Dienstmann, A. Patnaik, R. Garcia-Carbonero, A. Cervantes, M. Benavent, S. Roselló, B. B. Tops, R. S. van der Post, G. Argilés, N. J. 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).
 40. R. H. Whitehead, F. A. Macrae, D. J. St John, J. Ma, A colon cancer cell line (LIM1215) derived from a patient with inherited nonpolyposis colorectal cancer. *J Natl Cancer I* **74**, 759-765 (1985).
 41. 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).

42. A. Follenzi, L. Naldini, HIV-based vectors. Preparation and use. *Methods Mol Med* **69**, 259-274 (2002).
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. S. Li, K. R. Schmitz, P. D. Jeffrey, J. J. Wiltzius, P. Kussie, K. M. Ferguson, Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* **7**, 301-311 (2005).
45. 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. *Neoplasia* **14**, 1023-1031 (2012).

Acknowledgments: We thank the patients and their families. The authors thank members of the laboratory of Molecular Oncology at Candiolo Cancer Institute for critical reading and editing of this article. **Funding:** This study was supported by 5 per mille 2009 MIUR—from Fondazione Piemontese per la Ricerca sul Cancro—ONLUS ('Farmacogenomica' to FDN); Associazione Italiana per la Ricerca sul Cancro (AIRC) MFAG (11349 to FDN). European Community's Seventh Framework Programme under grant agreement no. 602901 MErCuRIC (A.Bardelli and F.D.N.); IMI contract n. 115749 CANCER-ID (A.Bardelli); AIRC 2010 Special Program Molecular Clinical Oncology 5 per mille, Project n. 9970 (A.Bardelli); Fondazione Piemontese per la Ricerca sul Cancro-ONLUS 5 per mille 2010 e 2011 Ministero della Salute (A.Bardelli and F.D.N.); Ministero dell'Istruzione, dell'Università e

della Ricerca - progetto PRIN 2010-2011 (A.Bardelli). **Author contributions:** S.A. and A.Bardelli designed and supervised the study. A.A.A. and R.N. followed patients and provided clinical samples. S.A., G.S. and B.M. performed experiments. A.B., S.A., G.S., C.M. and J.D.K. analyzed data. B.B.W., A. Bertotti, L.T., S.M. and L.L. provided reagents. S.A, F.D.N. and A.Bardelli wrote the manuscript. All authors reviewed the final manuscript. **Competing interests:** A. Bardelli is a consultant/advisory board member for Biocartis, Horizon Discovery, and Trovogene. C. M. is a consultant/advisory board member for Merck. R.N., B.B.W., and J.D.K. were employees of Merrimack Pharmaceuticals at the time of their contributions. A.A.A. is study investigator and consultant/advisory board member for Merrimack. The other authors declare no competing interests. **Data and materials availability:** LIM1215 can be obtained through materials transfer agreement from the Ludwig Institute for Cancer Research (Zurich, Switzerland). HCA-46 and CCK-81 cells are commercially available respectively from the European Collection of Cell Cultures (ECACC catalog no. 07031601) and from JCRB Cell Bank (JCRB0208). The HEK-293 and HEK-293T cell lines are commercially available from ATCC (CRL-1573 and CRL-3216) (LGC Standards S.r.l). Resistant cell lines can be obtained from the corresponding authors through material transfer agreement.

Table and Figures:

Subject ID	Study Treatment	EGFR ECD mutations
005-30-008	MM-151 Monotherapy	ND
010-30-007	MM-151 Monotherapy	ND
010-42-020	MM-151 Monotherapy	ND
054-62-051	MM-151 Monotherapy	<i>EGFR</i> p.S464L <i>EGFR</i> p.465R
065-72-065	MM-151 Monotherapy	ND
054-92-095	MM-151 Monotherapy	<i>EGFR</i> p.465E <i>EGFR</i> p.465R
065-92-103	MM-151 Monotherapy	ND
005-02-109	MM-151 Monotherapy	ND
065-02-093	MM-151 Monotherapy	ND
054-03-106	MM-151 + Irinotecan	ND
010-03-086	MM-151 + Irinotecan	ND

Table 1. Identification of EGFR ECD mutations in circulating cell-free tumor DNA of CRC patients. Serum samples were collected and analyzed by ddPCR in a

subset (N=11) of patients enrolled in the phase 1 clinical trial NCT01520389. Patients received MM-151 after emergence of acquired resistance to previous anti-EGFR treatment. The *EGFR* ECD mutations detected at baseline are noted for each patient. ND, not detected.

Figure 1. MM-151 engages three epitopes on the EGFR extracellular region and binds all *EGFR* ectodomain mutants. (A) Schematic representation of the four EGFR extracellular domains (sEGFR) derived from PDB structure “1NQL”(43). Highlighted in red are six amino acid positions identified as mutated in cetuximab-resistant tumors (EGFR p.R451C, p.S464L, p.G465E/R, p.K467T, p.I491M, p.S492R). Approximate binding sites for cetuximab, panitumumab, and MM-151 are indicated on the basis of published data(22, 44, 45). (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 5 µg/ml of cetuximab, panitumumab, or MM-151 and Halotag-EGF (tracer) at a concentration of 18 ng/ml for 30 minutes. The BRET ratio was normalized to the ligand-only control. Each experiment was repeated at least two times with duplicate replicates. (C) Cell proliferation assay performed on a panel of LIM1215 colorectal cancer cells engineered to express the indicated EGFR ectodomain mutants. Cells were treated for six 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 the 5 µg/ml drug concentration and as a drug titration series in **Fig. S3**. The experiment was repeated three times, with mean ± s.d. indicated. Cetux:

cetuximab; Panit: panitumumab. Statistical differences were calculated by unpaired Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001. Detailed data and p values provided in table S1 and S2.

Figure 2. MM-151 inhibits EGFR downstream signaling in cells expressing EGFR ectodomain mutants. 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).

A **B** **C** **D** **E** **F**

Figure 3. MM-151 inhibits proliferation and downstream signaling in preclinical models of acquired resistance to cetuximab driven by *EGFR* ECD mutations. (A-C) Effects of cetuximab, panitumumab, and MM-151 on EGFR-dependent signaling in cells that spontaneously developed EGFR ECD mutations (LIM1215, CCK-81, and HCA-46). 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 G456E mutation in the PDX and in the derivative cell line are indicated. (E) Cells derived from a PDX carrying the *EGFR* p.G465E variant (CRC G456E-XL) were treated for six days with increasing concentrations of cetuximab, panitumumab, and MM-151. Cell viability was measured by the adenosine triphosphate (ATP) assay. The experiment was repeated three times, with mean $\pm$ s.d. 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.

Figure 4. Monitoring mutant *EGFR* in liquid biopsies of patients during MM-151 treatment. (A,C) *EGFR* extracellular domain mutations were detected by ddPCR in cetuximab-refractory patients 051 and 095 at baseline before MM-151 treatment. The allelic frequency of these mutations declined in ctDNA during MM-151 treatment until progression occurred. **(B,D)** CT scans showing the 29% tumor size

reduction in patient 095 and disease stabilization in patient 051 during MM-151 treatment.