Amplification of the MET Receptor Drives Resistance to Anti-EGFR Therapies in Colorectal Cancer

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INTRODUCTION

Drugs targeting the EGF receptor (EGFR)—antibodies binding the extracellular domain and small-molecule tyrosine kinase inhibitors—have expanded treatment options for several solid tumors (1). The EGFR-targeted monoclonal antibodies cetuximab and panitumumab have been extensively studied in metastatic colorectal cancer, whereas tyrosine kinase inhibitors have thus far shown weak or no activity in this setting (2–4). Cetuximab or panitumumab seem to have similar therapeutic efficacy, achieving fairly modest but clinically meaningful objective (approximately 10%) response rates when used as monotherapy in genetically unselected patients with chemotherapy-refractory, EGFR-expressing metastatic colorectal cancer (5, 6).

It has been clearly established that KRAS mutational status is the key predictor of tumor suitability for anti-EGFR therapy (7, 8). As KRAS is a downstream component of the EGFR signaling pathway, cells with mutant KRAS do not respond to anti-EGFR therapies. BRAF mutations, which are mutually exclusive with KRAS, have also been associated with a lack of response to cetuximab and panitumumab (9). Deregulation of other effectors of the EGFR signaling cascade (PIK3CA, PTEN, NRAS, etc.) or of EGFR modulators (HER2, EGFR ligands, etc.) is also thought to affect primary response to EGFR blockade (10–12). Altogether, these primary mechanisms of resistance account for 70% to 80% of the cases unresponsive to anti-EGFR therapies, suggesting that there might be additional, yet undiscovered, biomarkers of resistance to these agents.

Importantly, the clinical efficacy of EGFR-targeted antibodies is limited by the development of acquired (secondary) resistance, which typically occurs within 3 to 12 months of starting therapy (5, 6). Further therapeutic options for these patients are very limited. Understanding the molecular bases of relapse to EGFR blockade in colorectal cancer is therefore clearly relevant to developing novel therapeutic strategies.

Multiple mechanisms of secondary resistance to anti-EGFR antibodies have been reported, such as expression of EGFR ligands, HER2 amplification, and deregulation of the EGFR recycling process (12–16). We recently discovered that secondary KRAS mutations arise and are responsible for acquired resistance in approximately 50% of the patients who initially respond to cetuximab or panitumumab (17, 18). KRAS-mutant alleles can be detected in patients’ blood using highly sensitive circulating tumor DNA analysis methods before disease progression is clinically manifested (17, 18). In the present work, we have studied the molecular bases of relapse in those patients who do not develop KRAS mutations during the course of anti-EGFR therapy.

RESULTS

MET Amplification Is Associated with Acquired Resistance to Cetuximab or Panitumumab in Metastatic Colorectal Cancer Patients

We analyzed 7 patients with colorectal cancer who initially responded to panitumumab- or cetuximab-based treatment...
and then relapsed (Table 1). Of these, 4 did not display KRAS mutations in plasma samples analyzed by the highly sensitive BEAMing (Beads, Emulsions, Amplification, and Magnetics) technique (18). For 3 of these patients (#1, #2, and #3; Table 1) tumor tissue—before and after anti-EGFR therapy—was available through surgical or biopotic procedures. Genomic DNA (gDNA) extracted from these cases was subjected to exome sequencing and next-generation Digital Karyotyping analyses with the aim of identifying sequence and copy number alterations present only in the post-relapse tissue. In all 3 cases, in the tissue obtained after anti-EGFR treatment, we detected amplification of a genomic fragment encompassing the MET gene, encoding the tyrosine kinase receptor for hepatocyte growth factor (HGF). Quantitative PCR (qPCR) analysis confirmed the presence of MET amplification in the post-therapy samples but not in the matched pretreatment tissues (Fig. 1). The absence of KRAS mutations was verified in both pre- and post-tissues, thus confirming the analyses conducted in blood (data not shown). Mutations in other genes known to be involved in EGFR signaling (such as BRAF, NRAS, and PIK3CA) were also excluded by next-generation sequencing (NGS; data not shown). FISH analysis confirmed MET amplification (see Methods for details) in the samples of patients #1, #2, and #3 obtained at relapse (Fig. 2). FISH analysis showed that MET was not amplified in the tumor tissue obtained before anti-EGFR treatment for patients #1 and #2 (Fig. 2A and B); however, it revealed the presence of rare MET-amplified cells in the sample from patient #3 obtained before treatment with cetuximab (Fig. 2C). At least in this instance, we can therefore hypothesize that EGFR-targeted therapies acted as a selective pressure to expand a preexisting minor subclonal population of cancer cells carrying MET amplification. Immunohistochemistry (IHC) was then used to assess whether MET amplification translated into overexpression of the MET receptor. Stronger MET immunostaining was present in the postrelapse compared with the prerelapse tissue (Fig. 2). In an additional patient (#4), where exome analyses could not be conducted because of the low amount of material retrieved by the biopotic procedure upon relapse, we were able to exclude the presence of genetic alterations in genes previously implicated with primary resistance to anti-EGFR therapies (KRAS, BRAF, NRAS, HRAS, PIK3CA, EGFR, and HER2; data not shown). Because in patient #4 FISH and IHC ruled out MET amplification or overexpression (data not shown), the mechanisms of acquired resistance to anti-EGFR therapy remain to be elucidated. Finally, IHC showed that the levels of MET expression were low or undetectable in the postrelapse tissue samples of patients #5, #6, and #7 that displayed KRAS mutations (Supplementary Fig. S1).

Noninvasive Monitoring of MET Amplification in Blood Samples

We reasoned that a genetic-based strategy to specifically detect MET gene amplification would allow us to assess whether this event was already present in a subset of the tumor cells before anti-EGFR therapy was initiated. In principle, such a biomarker could also be used to noninvasively monitor the emergence of MET-driven secondary resistance to anti-EGFR therapy in circulating tumor DNA from blood samples.

We initially used real-time PCR to detect increased gene copy number in DNA extracted from the plasma of patients

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Treatment</th>
<th>Duration of treatment</th>
<th>Pre-EGFR therapy sample</th>
<th>Post-EGFR therapy sample</th>
<th># KRAS mutant alleles/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Panitumumab</td>
<td>12 mo (Aug 2010 to Aug 2011)</td>
<td>Para-aortic lymphodes: KRAS wt, MET wt</td>
<td>Liver metastasis: MET ampl, KRAS wt</td>
<td>Plasma ctDNA: KRAS wt (0/16,900 events)</td>
</tr>
<tr>
<td>#2</td>
<td>Panitumumab</td>
<td>13 mo (Mar 2010 to Apr 2011)</td>
<td>Liver metastasis: KRAS wt, MET wt</td>
<td>Liver metastasis: MET ampl, KRAS wt</td>
<td>Plasma ctDNA: KRAS wt (0/126,700 events)</td>
</tr>
<tr>
<td>#3</td>
<td>Cetuximab+ irinotecan</td>
<td>12 mo (Feb 2011 to Feb 2012)</td>
<td>Liver metastasis: KRAS wt, MET wt</td>
<td>Lung metastasis: MET ampl, KRAS wt</td>
<td>Plasma ctDNA: KRAS wt (0/11,600 events)</td>
</tr>
<tr>
<td>#4</td>
<td>Cetuximab+ irinotecan</td>
<td>6 mo (Mar 2011 to Sep 2011)</td>
<td>Abdominal lymphode: KRAS wt, MET n.a.</td>
<td>Liver metastasis: KRAS wt, MET wt</td>
<td>Plasma ctDNA not available</td>
</tr>
</tbody>
</table>

*Patient #3 received cetuximab monotherapy from May 2011 to February 2012 (after receiving cetuximab + irinotecan from February to May 2011).  
*The preexistence of single and rare MET-amplified cells was observed by FISH analysis in the pre-cetuximab sample from patient #3.  
*KRAS was found to be wild-type in the DNA from the liver metastatic biopsy obtained after anti-EGFR treatment (0 of 165,000 events).  
Abbreviations: ctDNA, circulating tumor DNA; wt, wild-type; n.a., not available.
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Figure 1. Whole-exome analysis reveals increased MET copy number in colorectal cancer samples from patients who developed resistance to anti-EGFR treatment. A–C, left, whole-exome gene copy number (GCN) analysis of colorectal cancer tumor samples from 3 patients taken before (blue) and after (red) therapy with the EGFR-targeted monoclonal antibodies panitumumab (Pmab; patients #1 and #2) or cetuximab (Cmab; patient #3). Individual chromosomes are indicated on the x-axis. The lines indicate the sequencing depth as copy number values relative to a diploid exome (y-axis) over windows of 500,000 base pairs. A–C, right, MET amplification was confirmed in the paired tumor samples by qPCR gene copy number analysis. MAD1L1, reference gene on Chr.7.

with or without amplification of the MET gene. Although this approach could readily detect MET gene amplification in DNA extracted from cancer cell lines or tissues (Fig. 1), it was not successful using plasma-derived DNA. We hypothesized that this was due to the limited specificity and sensitivity of this approach when applied to circulating cell-free DNA, which is composed of a mixture of tumor and normal nucleic acids. Indeed, while somatic point mutations (such as those in KRAS) are tumor-specific, increased dosage of wild-type loci is highly affected by the concomitant presence of the circulating normal DNA. We reasoned that genetic events occurring concomitantly with intra- or extrachromosomal DNA amplification (19, 20) might be exploited as unique (tumor-specific) genetic identifiers. As these molecular events often occur within noncoding regions, we extended the exome analysis by conducting whole-genome sequencing on the post-relapse tumor.

As a test case, we analyzed patient #2, for whom tumor tissue obtained before and after anti-EGFR therapy as well as a longitudinal collection of blood samples obtained while on therapy and at relapse were available. Using an algorithm designed to identify amplification-associated chromosomal rearrangements (20–22), we retrieved sequencing reads encompassing 2 noncontiguous loci (surrounding the amplified region; see Supplementary Fig. S2).

Next, PCR primers were designed to detect the presence of this rearrangement (which represents the genomic...
breakpoint associated with the amplification of the MET gene as an 89-bp tumor-specific PCR product. We also designed a control assay that encompassed the same locus generating a 124-bp product that was not affected by the presence of MET amplification (Supplementary Fig. S2).

The 89-bp product specific to the MET-amplified rearrangement was not present in control DNAs obtained from a colorectal cancer cell line and in the germline DNA [peripheral blood mononuclear cell (PBMC) derived] of patient #2, but it was detected at high levels in the postrelapse tissue (Fig. 3). Interestingly, very low levels of the MET amplification-associated chromosomal rearrangement were already present in the surgical specimen obtained before initiation of the anti-EGFR therapy (Fig. 3). Using the same approach, we analyzed DNA extracted from plasma samples obtained at 3-month intervals from the initiation of EGFR therapy (Fig. 3). Using the MET amplification-specific product was evident in the blood of this patient before relapse as determined by computed tomography (CT) scan, suggesting that detection of MET amplification may provide a highly sensitive method for monitoring molecular resistance and recurrence in this setting. The same approach was then applied to patient #3. Also, in this case a genomic breakpoint associated with the amplification of the MET gene could be identified by whole-genome analysis of postrelapse tissue (Supplementary Fig. S3). PCR primers were then designed and used to detect a MET amplification-associated chromosomal rearrangement in the post-therapy samples (tissue and plasma) of patient #3 (Supplementary Fig. S3, bottom).

**MET Amplification Is Associated with Primary Resistance to Cetuximab in Colorectal Cancer Patient-Derived Tumor Xenografts (“Xenopatients”)**

KRAS mutations drive both primary and secondary (acquired) resistance to cetuximab and panitumumab. We reasoned that MET gene amplification could also be responsible for primary resistance to EGFR-targeted antibodies in colorectal cancer. To assess this hypothesis, we took advantage of a large collection of patient-derived colorectal cancer liver metastasis xenografts (“xenopatients”), which we previously annotated for their molecular profile and sensitivity to cetuximab (11). The metastatic colorectal cancer xenopatients responded to cetuximab with rates and extents analogous to those observed in the clinic: approximately 10% of the cases displayed partial response (PR) and 30% had disease stabilization, whereas 60% progressed on anti-EGFR therapy (11). We previously reported that, in agreement with what was found in the clinical setting, mutations in KRAS, BRAF, NRAS, or PIK3CA and amplification of HER2 are associated with resistance to cetuximab in colorectal cancer xenopatients (10–12). Overall, these biomarkers account for most but not all of the samples in which the anti-EGFR therapy was ineffective. We reasoned that MET amplification could be responsible for some of these unexplained samples and would be mutually exclusive with the other genetic lesions.

By extending the analyses previously conducted on a subset of samples (23), we assessed the MET gene copy status in the entire xenopatient cohort and found that 1% (2 of 196) of cases carried MET amplification, as determined by real-time
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**Figure 3.** Monitoring MET amplification in circulating tumor DNA during anti-EGFR therapy. A, size of liver metastases at segment 1 (S1; red), segments 2 to 3 (S2–S3; green), and segment 5 (S5; violet) and of lymph node target lesions at the hepatoduodenal ligament (cyan) during panitumumab (Pmab) therapy in patient #2 at the indicated time points, showing response to panitumumab followed by progression. B, carcinoembryonic antigen (CEA) levels (blue line) and number of genome equivalent (GE; red line) obtained from 1 mL of plasma from patient #2, as assessed by real-time PCR. C, DNA electrophoresis of PCR products using primers designed to detect the presence of the MET-associated amplified rearrangement on chromosome 7. The bottom band corresponds to an 89-bp tumor-specific PCR product, which is positive only when the rearrangement is present. A control assay detecting the wild-type locus generated amplicon of 124-bp (top band) is also shown.

PCR analysis (Fig. 4A). Importantly, amplification of the MET gene and overexpression of the MET protein were confirmed by FISH and IHC, respectively, in both xenografted tumors and their original human counterparts (Fig. 4B). Notably, none of the patients with metastatic colorectal cancer from whom the “xenopatients” were derived had been exposed to cetuximab or panitumumab, ruling out the possibility that MET amplification could have been positively selected by previous anti-EGFR therapy. Interestingly, treatment with cetuximab was ineffective in mice engrafted with colorectal cancer metastatic specimens carrying MET amplification (Fig. 4C).

Indeed, the MET-amplified cases segregated into the subpopulation of xenopatients resistant to cetuximab and wild-type for KRAS, NRAS, BRAF, PIK3CA, and HER2 (16 cases; Fig. 4D). Overall, these data indicate that MET amplification characterizes a significant fraction (2 of 16, 12.5%; Fig. 4D) of cetuximab-resistant cases that are wild-type for KRAS, NRAS, PIK3CA, and HER2, possibly identifying a new biologically distinct metastatic colorectal cancer subpopulation ($P = 0.006$ by Fisher’s exact test).
Figure 4. MET amplification is associated with lack of response to cetuximab in a series of colorectal cancer (CRC) patient-derived xenografts (“xenopatients”). A, qPCR gene copy number analysis of MET amplification in a series of cetuximab-resistant “xenopatients,” which did not carry genetic alterations in genes previously associated with resistance to anti-EGFR therapy (KRAS, BRAF, NRAS, PIK3CA, and HER2). Dotted line indicates an estimated copy number of 2. B, FISH and IHC analysis of MET in colorectal cancer samples that showed increased MET copy number by qPCR analysis. Patients (left) and corresponding xenopatients (right) are shown. MET (7q31), red; D7Z1 chromosome 7 centromeric probe (7p11.1–q11.1), green. FISH, original magnification ×60. IHC, original magnification ×40. C, growth curves in mice cohorts derived from MET-amplified xenopatients, treated with placebo (blue) or cetuximab 20 mg/kg i.p. twice a week (red). n = 6 for each treatment arm. Arrows indicate treatment start. D, prevalence of MET amplification in unselected metastatic colorectal cancer samples, according to qPCR experiments (left), and in cetuximab-resistant (Cmab), genetically selected (without genetic alterations in KRAS, BRAF, NRAS, PIK3CA, and HER2) xenopatients (right). P values were calculated by Fisher’s exact test. wt, wild-type.
MET Activation Drives Resistance to EGFR Inhibitors in Preclinical Colorectal Cancer Models

Data obtained in patients’ samples and in xenografted tumors suggest that amplification of the MET locus, sustaining overexpression of the MET receptor, mediates resistance to EGFR blockade in colorectal cancers. It has been previously established that overexpression constitutively activates the MET receptor and is an oncogenic event in multiple cancer types (24, 25). To formally assess whether MET overexpression alone is causally responsible for cetuximab or panitumumab resistance, we conducted in vitro and in vivo forward genetic experiments. As model systems, we explored 2 colorectal cancer cell lines, namely DiFi and LIM1215. DiFi cells overexpress EGFR as a result of high-level amplification of the EGFR gene locus (26, 27). In contrast, LIM1215 cells express “normal” EGFR levels but are still sensitive to cetuximab or panitumumab (28, 29). Both cell lines are wild-type for KRAS, BRAF, NRAS, and PIK3CA, paralleling the molecular features of the patients with colorectal cancer most likely to respond to cetuximab. Ectopic overexpression of the MET receptor was achieved in both cell lines by means of lentiviral-mediated transduction of the corresponding cDNA. As control, transduction with KRAS or a kinase-inactive version of MET (MET kinase dead) was used (Supplementary Fig. S4). As shown in Fig. 5A, MET constitutive activation, due to cDNA transfection and consequent protein overexpression, conferred resistance to cetuximab or panitumumab in proliferation assays to a degree equivalent to that triggered by KRAS. Notably, the ability of wild-type MET to drive resistance to anti-EGFR monoclonal antibodies was abolished by the concomitant treatment with the anti-MET inhibitor JNJ-38877605 (30), further confirming that MET-promoted intracellular signaling was driving resistance (Fig. 5A).

Another mechanism of MET-mediated oncogenic activation is represented by autocrine or paracrine stimulation by its ligand, HGF (31–34). Additional experiments were therefore carried out in DiFi and LIM1215 cells to assess whether HGF-induced MET activation could also endure resistance to cetuximab or panitumumab. The MET inhibitor JNJ-38877605 served as control. Indeed, paracrine activation of MET by HGF was sufficient to confer resistance to anti-EGFR antibodies (Fig. 5B).

These results suggest that HGF/MET-initiated signaling can bypass blockade of the EGFR by the monoclonal antibodies cetuximab and panitumumab. To get a mechanistic insight into how the HGF–MET ligand receptor pair could overcome EGFR inhibition, we carried out biochemical experiments. We found that HGF-mediated MET activation counteracted the cetuximab- and panitumumab-induced inhibition of mitogen-activated protein kinase (MAPK) and, to a lesser extent, of AKT (Fig. 5C).

To corroborate the results obtained in vitro, we carried out in vivo experiments in mouse models. Stable, ectopic transduction of the MET receptor was not conductive in DiFi and LIM1215, as transduced cells lost expression of the MET transgene after few passages. On the other hand, we found that long-term HGF overexpression was feasible and resulted in constitutive activation of the HGF/MET signaling axis in DiFi cells. Parental and HGF-expressing DiFi cells were therefore subcutaneously injected in immunocompromised mice and allowed to form palpable tumors. At this point, animals received cetuximab for 3 weeks, which, as expected, profoundly inhibited growth of the parental cells but had minimal or no influence on tumors expressing the MET ligand HGF (Fig. 5D). Cotreatment with cetuximab and the MET inhibitor JNJ-38877605 instead induced marked tumor regression.

These data suggest that colorectal cancers displaying constitutive activation of MET signaling triggered by either MET amplification or HGF-induced MET activation might be effectively targeted by MET inhibitors.

Patient-Derived Xenografts with MET Amplification Respond to MET Inhibitors

To ascertain the potential clinical relevance of the findings described earlier, we decided to adapt the intervention trial executed in colorectal cancer cell-line xenografts to patient-derived xenografts, which represent a more reliable proxy of prospective findings in patients. In this pilot study, we used 1 case of primary resistance (M162) and 1 model of secondary resistance (derived from the post-treatment tumor tissue of patient #3). As for the choice of therapeutic regimens, we focused on small-molecule inhibitors of MET that were administered individually or in combination with cetuximab. We selected JNJ-38877605, the MET-specific tool compound (not in clinical use) already used in the xenograft experiments with cell lines, and crizotinib, a dual MET/ALK inhibitor that has shown promising antitumor activity in MET-amplified esophagogastric adenocarcinomas (35). For each case, the original tumor specimen was serially passaged in vivo. When xenografts reached an average volume of approximately 500 mm³, mice were randomized into 6 independent treatment cohorts: (i) vehicle (placebo), (ii) cetuximab alone, (iii) JNJ-38877605 alone, (iv) crizotinib alone; (v) JNJ-38877605 and cetuximab, and (vi) crizotinib and cetuximab. In case M162 (primary resistance), crizotinib monotherapy produced initial shrinkage followed by slow resumption of tumor growth, which was substantially delayed by cotreatment with crizotinib (Fig. 6A). The efficacy of JNJ-38877605 was more pronounced, with long-lasting abolition of tumor growth even in the absence of cetuximab (Fig. 6A).

The response of patient #3 xenografts (a model of secondary resistance) to the same regimens was substantially analogous. In concordance with the results obtained in M162, all treatments potently delayed tumor growth. In contrast to M162, the antitumor activity of crizotinib was not enhanced by the addition of cetuximab, and the most effective modality in producing durable disease stabilization proved to be the JNJ-38877605–cetuximab combination (Fig. 6B).

Notwithstanding minor individual differences, these results overall indicate that interception of MET signaling leads to severe impairment of tumor growth in MET-amplified colorectal cancers and provides proof-of-concept for the use of MET inhibitors, alone or in combination with anti-EGFR antibodies, as novel therapeutic opportunities to contrast MET-driven primary and secondary resistance in the clinic.

DISCUSSION

Drugs directed against oncoproteins that sustain the growth of cancer cells have emerged as important therapeutic agents in
**Figure 5.** MET activation confers resistance to cetuximab (Cmab) or panitumumab (Pmab) in colon cancer cell lines in vitro and in vivo. 

A, DiFi and LIM1215 cell lines were transduced with the following lentiviral vectors: MOCK (empty vector), MET, MET KD (kinase dead), or KRAS, then seeded in 96-well plates and cultured 7 days in the presence of vehicle (NT), cetuximab (1 µg/mL) or panitumumab (1 µg/mL), with or without the MET inhibitor JNJ-38877605 (250 nmol/L).

B, DiFi and LIM1215 cells were treated for 1 week with increasing concentrations of cetuximab or panitumumab, with or without 20 ng/mL of HGF or HGF plus the MET inhibitor JNJ-38877605 (250 nmol/L).

C, DiFi and LIM1215 cells were pretreated 24 hours with cetuximab (1 µg/mL), panitumumab (1 µg/mL), or JNJ-38877605 (250 nmol/L), then stimulated for 15 minutes with HGF (80 ng/mL) in the presence of the indicated inhibitors. Whole-cell extracts were then subjected to Western blot analysis and probed with the indicated antibodies.

D, wild-type (wt) DiFi cells or DiFi transduced with HGF were subcutaneously injected in NOD/SCID mice. Upon tumor growth, mice were randomized (7 mice/group) and received intratumoral injection of saline/cetuximab or intratumoral injection of cetuximab and oral administration of JNJ-38877605. Arrows indicate treatment start.
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Among these, inhibitors of receptor tyrosine kinases (antibodies or small molecules) have shown marked clinical activity (1). Unfortunately, the overall value of these agents is substantially limited by the acquisition of drug resistance, which eventually arises in most, if not all, treated patients. Several hypotheses have been put forward to explain why resistance arises. One well-supported possibility is that the lesions which respond to the treatment are genetically heterogeneous and already contain a large selection of molecular variants from which the drugs simply select those conferring resistance in a classic Darwinian fashion (reviewed in ref. 36).

In this work, we have studied the mechanisms of acquired resistance to cetuximab and panitumumab, 2 monoclonal antibodies that inhibit the signaling cascade initiated by the EGFR receptor and in the clinical setting ameliorate the survival of patients with metastatic colorectal cancer. Similar to what happens with other targeted agents, patients with metastatic colorectal cancer whose tumors respond to EGFR-targeted antibodies develop acquired resistance within 3 to 12 months (5, 6). We have recently reported that resistance in this setting can be driven by the selection of oncogenic mutations in the KRAS gene or, less frequently, amplification of the KRAS locus (18). Using a highly sensitive digital PCR approach (BEAMing), we were able to detect the emergence of KRAS mutations in patients’ blood months before relapse was evidenced by CT scans. Analysis of plasma samples from patients receiving panitumumab monotherapy likewise showed the emergence of oncogenic KRAS variants in approximately 40% of the cases (17). Together, these analyses suggest that in a large proportion of patients with metastatic colorectal cancer who respond and then become refractory to cetuximab or panitumumab, resistance is caused by the emergence of KRAS oncogenic alleles. At the same time, they raise the question of which molecular mechanisms drive resistance in the remaining patients.

To tackle this, we used BEAMing to select patients in whom KRAS mutations did not emerge during therapy. To investigate novel mechanisms of resistance, gDNA was extracted from 3 cases and subjected to exome sequencing alongside the corresponding pretreatment neoplastic tissue. High levels of MET amplification were found at relapse. No...
other gene copy number variations were detected and NGS analysis confirmed that other candidate drivers of resistance (KRAS, NRAS, BRAF, and PIK3CA) remained wild-type. Post-relapse tissue was also analyzed by FISH, which confirmed MET amplification, and by IHC, which showed high levels of MET protein expression.

The MET gene, encoding the tyrosine kinase receptor for HGF, has been shown to have an oncogenic role in several human tumors, where it becomes constitutively activated as a consequence of gene amplification, overexpression, activating mutations, or autocrine stimulation (37). MET and its ligand HGF have been previously implicated in acquired resistance to targeted therapies. For example, MET amplification is found in approximately 5% to 20% of the EGFR-mutated lung cancers that respond and then progress on erlotinib/gefitinib-based therapies (38–40). Notably, MET amplification seems to arise in tumors with preexisting clones of MET-amplified cells, which undergo positive selection during gefitinib and erlotinib therapy (41). The same seems to be true for patient #3 in our cohort, as FISH analyses identified the presence of rare MET-amplified cells in the sample before cetuximab exposure.

If patients with metastatic colorectal cancer became resistant to anti-EGFR antibodies as a result of the emergence of MET amplification in their tumors, we expected that this genetic event could be detected in their circulation during the therapy. To assess this possibility, we developed a PCR-based assay to detect the presence of the MET amplicon in circulating, cell-free DNA. The test was applied to longitudinal blood samples from patient #2 and showed that MET amplification was present as early as 3 months after initiation of therapy. As a blood draw before anti-EGFR therapy was not available for this patient, we could not assess the status of the MET amplicon in the plasma at baseline. Notably, however, when the same analysis was applied to the pretreatment tissue, MET amplification was detected although at low level. These findings relative to patient #2, together with the IHC and FISH analyses conducted on the pretreatment tissue of patient #3, support the hypothesis that anti-EGFR therapy selects MET-amplified (cetuximab- and panitumumab-resistant) preexisting clones. During treatment, MET-amplified cells would then become the leading population, eventually limiting the efficacy of further anti-EGFR therapies. If confirmed in larger datasets, these results support the use of blood tests to monitor the emergence of MET amplification in patients undergoing anti-EGFR therapies. This approach may drive the early initiation of MET inhibitors in those patients who respond to cetuximab and panitumumab and do not display emergence of KRAS mutations in blood tests during anti-EGFR therapy.

The discovery of MET amplification as a mechanism of secondary resistance to cetuximab prompted us to hypothesize that the same genetic alteration might be responsible for de novo resistance to EGFR-targeted antibodies in colorectal cancers. Indeed, we found that xenografted tumors (“xenopatients”) carrying MET amplification did not respond to cetuximab and that this molecular alteration was mutually exclusive with mutations in KRAS, BRAF, NRAS, PIK3CA, and with HER2 amplification. It should be noted that the prevalence of MET amplification in untreated metastatic colorectal cancer was low—around 1% (2 of 196 samples) in our cohort, consistent with the frequency (1 of 193 cases) reported by the recent The Cancer Genome Atlas consortium (42), as well as in previous studies (43, 44). For this reason, as described for other low-prevalence driver alterations (10, 45), the clinical validation of MET amplification as a biomarker of resistance to anti-EGFR therapy in metastatic colorectal cancer will require very large retrospective (and possibly prospective) studies.

Cells of epithelial–endothelial origin widely express the MET receptor, which is essential for embryonic development and tissue repair. HGF, the ligand for the MET receptor, is expressed mainly in cells of mesenchymal origin, although some tumors seem to express both HGF and MET (25). Whether amplification-driven MET overexpression is sufficient to fully activate its oncogenic properties remains a controversial matter. In general, it seems that the addition of HGF can further trigger MET-initiated signaling. Our functional analysis indeed indicates that HGF plays an important role in driving MET-mediated resistance to anti-EGFR monoclonal antibodies in colorectal cancer cells. Furthermore, in agreement with recent studies (29, 46, 47), we found that HGF stimulation is sufficient to confer cetuximab and panitumumab resistance both in vitro and in vivo. These findings support the possibility that HGF overexpression by cancer cells or the surrounding stroma might be an independent mechanism of acquired (or primary) resistance to cetuximab. This could be particularly relevant in patients with hepatic metastasis, as the liver is a known reservoir for HGF (48).

The most relevant aspect of our work is that MET (as opposed to KRAS) is an actionable target. Multiple agents, some of which are already approved for clinical use, have been developed to target MET or HGF, including crizotinib, which was originally designed to inhibit MET and only later found to be active for patients with mutant ALK or ROS-1 (30, 49). HGF-directed antibodies are also being evaluated in clinical trials with some encouraging early results (30). The preclinical trial in which we treated 2 colorectal cancer xenopatients carrying MET amplification is encouraging. In both instances Met inhibitors (including the clinically approved drug crizotinib) were effective. Our results therefore support the initiation of clinical trials based on MET inhibition in the subgroup of patients with colorectal cancer with MET amplification (de novo or acquired).

Importantly, one of the treated xenopatients was obtained from a biopsy (patient #3). This individual is currently alive, further underscoring the translational impact of these results.

Because of limited tissue availability, we have been unable to conduct whole-genome analysis and identify putative mechanisms of resistance in 1 of 7 patients (patient #4) analyzed in this study. We confirmed the absence of MET gene amplification by FISH, and a candidate-biomarker approach ruled out the presence of mutations or amplifications in known EGFR pathway components (KRAS, BRAF, NRAS, HRAS, PIK3CA, EGFR, and HER2). It is therefore likely that mechanisms other than KRAS or MET oncogenic alterations could be responsible for the emergence of resistance to EGFR antagonists in a fraction of metastatic colorectal cancer cases. On the basis of our current finding, we forecast that this additional mechanism will be based on the deregulation of genes involved in EGFR signaling that have been previously associated with “de novo” resistance to anti-EGFR therapies.

When considered together, KRAS mutations or MET amplification occur in the large majority of patients with
metastatic colorectal cancer who initially respond and then relapse on anti-EGFR therapies and likely represent major mechanisms of acquired resistance to anti-EGFR therapies. Current evidence suggests that these 2 molecular alterations occur in a mutually exclusive fashion, thus defining independent patient populations that will likely require different therapeutic approaches once resistance ensues. Nevertheless, our findings do not rule out the possibility that within the same patient distinct metastatic lesions might evolve independent resistance mechanisms. In conclusion, this work defines MET gene amplification as a novel mechanism of both primary and acquired resistance to cetuximab or panitumumab and identifies a patient population that could immediately benefit from the clinically available MET inhibitors.

METHODS

Patients and Tumor Samples

We retrospectively analyzed 7 patients with histologically confirmed metastatic colorectal cancer at Ospedale Niguarda Ca’ Granda (Milan, Italy). Tumor specimens were obtained through protocols approved by the Institutional Review Board of Ospedale Niguarda Ca’ Granda (protocols 1014/2009 and 194/2010). All tumor samples were formalin-fixed paraffin-embedded (FFPE). All patients provided informed consent, and samples were procured and the study was conducted under the approval of the Review Boards and Ethical Committees of the Institutions. Patients evaluated in this study were selected on the basis of evidence that treatment outcome could most likely be attributable to administration of either panitumumab or cetuximab (synergy with irinotecan should be taken into account for those patients treated with cetuximab in combination with irinotecan in the chemorefractory setting). Patients were enrolled in clinical trials or received panitumumab or cetuximab as per label indication. For those patients who were treated with cetuximab in combination with irinotecan, refractoriness to previous irinotecan-based regimens was documented as disease progression during, or within, 6 months of receiving the irinotecan-based regimen (administered for at least 6 weeks). Besides the abovementioned inclusion criteria, the availability of tumor sample quantitatively and qualitatively suitable for molecular analyses was also a requirement for being considered in the present study. Clinical response was assessed every 6 to 8 weeks with radiologic examination (CT or MRI). The Response Evaluation Criteria in Solid Tumors (50) were adopted for evaluation, and objective tumor response was classified into PR, stable disease, and progressive disease (PD). Patients with stable disease or PD were defined as non-responders (50). Two independent oncologists and radiologists verified in a blinded manner the clinical response for all patients.

Plasma Samples Collection

At least 4 mL of whole blood were collected by blood draw using EDTA as anticoagulant. Plasma was separated within 5 hours using EDTA as anticoagulant. Plasma was separated within 5 hours (the first at room temperature for 10 minutes at 1,600 × g and the second at 3,000 × g for the same time and temperature), obtaining 1 mL of plasma. Plasma was stored at −80°C until DNA extraction.

DNA Extraction

Tissue sections of 5-μm thickness were obtained from FFPE tissues and stained with hematoxylin. To enrich for malignant cells, neoplastic areas were macro/microdissected from tissue slides by scraping under microscopic guidance. gDNA was extracted with QIAamp DNA FFPE tissue kit (Qiagen) according to the manufacturer’s protocol, and relative concentration was quantified using the Infinite 200 NanoQuant spectrophotometer (Tecan). Plasma was thawed at room temperature and centrifuged at 16,000 × g for 5 minutes to remove any cell debris. DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen), using columns with silica-based membrane, tube extenders, and a vacuum pump, according to manufacturer’s instructions. The DNA was eluted in 2 steps with 140 μL RNase-free water. DNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The average concentration was 50 ng/μL, with 260:280 and 260:230 ratios around 2.70 and 0.45, respectively.

Cancer Exome and Genome Sequence Analyses and Digital Karyotyping

Library construction, exome capture, NGS, and bioinformatic analyses of tumor and normal samples were conducted at Personal Genome Diagnostics (Baltimore, MD). In brief, gDNA from tumor and normal samples were fragmented and used for Illumina TrueSeq library construction (Illumina). Exonic regions were captured in solution using the Agilent SureSelect 50 Mb kit (version 4) according to the manufacturer’s instructions (Agilent). Paired-end sequencing of both exome and genome libraries, resulting in 100 bases from each end of the fragments, was conducted using a HiSeq 2000 Genome Analyzer (Illumina). The tags were aligned to the human genome reference sequence (hg18) using the Eland algorithm of CASAVA 1.7 software (Illumina). The chastity filter of the Basecall software of Illumina was used to select sequence reads for subsequent analysis. The ELANDv2 algorithm of CASAVA 1.7 software (Illumina) was then applied to identify point mutations and small insertions and deletions. Known sequence polymorphisms recorded in the single-nucleotide polymorphism database (dbSNP) were removed from the analysis. Potential somatic mutations were filtered and visually inspected as described previously (22). Copy number analyses using next-generation Digital Karyotyping, and rearrangement analyses were conducted as previously described (20–22).

Gene Copy Number Analysis (NGS)

Reads’ depths for both samples were calculated filtering the samtools mpileup results for positions actually included in coding exons of RefSeq according to hg18. Averages over overlapping 500,000 bp wide windows were then computed and plotted.

Real-Time PCR for circulating DNA Quantification

The extracted circulating DNA was quantified with a real-time PCR using Human LINE quantification. PCR is conducted in a final volume of 10 μL, containing 5 μL of SYBR Mix (Promega), 0.72 μL of 12.5 μmol/L for each forward and reverse LINE primer (forward primer: 5′-TCACTCAAAAGGGCTCTAATC-3′; reverse primer: 5′-TCTGCTTCTTTGTTATG-3′), 0.56 μL of water, and 3 μL of DNA. All reactions are carried out in triplicate. Various dilutions of normal human DNA purified from human colonocytes were incorporated in each plate to serve as standards. The analysis of the results obtained by real-time PCR is used to calculate the number of genome equivalents present in 1 mL of each sample of plasma.

Endpoint PCR

The amplification was conducted in a 10 μL PCR reaction, with 2× Phusion Flash PCR Master Mix (NEB, BioLabs) and 0.5 μmol/L of each primer (forward: 5′-gaaagagtgaatagcctg-3′; reverse: 5′-ccctcttccgtgctctc-3′; LIC: 5′-ggatatagcctagcctg-3′). Amplification was carried out using the following cycling conditions: 98°C for 120 seconds; 3 cycles of 98°C for 10 seconds, 69°C for 15 seconds, 72°C for 15 seconds; 3 cycles of 98°C for 10 seconds,
of a 96-well PCR plate together with 70 nM of each deoxynucleoside triphosphate, and 0.5 mmol/L MgCl₂. The water-in-oil emulsion was obtained by pipetting. Amplification was conducted in a 50 μL PCR reactions were distributed into the wells of the p-arm of chromosome 7, was evaluated for Copy Number Variation (CNV). PCR runs were conducted using ABI MicroAmp Optical Fast 96-well Reaction Plates on an Applied Biosystems ViiA 7 Real-Time PCR System (Applied Biosystems) using 20 ng total gDNA as a template. CNV of the target gene was calculated by the ABI SDS software 1.1 using relative quantification based on the ΔΔCt method and control samples as calibrators.

Chemical Treatment and Inhibitors

LIM1215 cells were grown in RPMI-1640 medium with 10% FBS and 1 μg/mL insulin. The DiFi cell line was cultured in F12 medium supplemented with 10% FBS. DiFi were a kind gift from Dr. J. Baselga (Oncology Department, Vall d’Hebron University Hospital, Barcelona, Spain) in November 2004, whereas LIM1215 were obtained from R. Whitehead (Vanderbilt University, Nashville, Tennessee; ref. 51), with permission from the Ludwig Institute for Cancer Research (Zurich, Switzerland) in November 2009. The genetic identity of the cell lines was confirmed by short tandem repeat profiling (Cell ID; Promega), which was last repeated in January 2013. HGF was from Peprotech. JNJ-38877605 was provided by Janssen, cetuximab and panitumumab were obtained from the Pharmacy at Niguarda Ca’ Granda Hospital (Milan, Italy). MET, MET kinase dead, and KRAS constructs were in the p156RRLaIn. PPTiCMV.MCS pre lentiviral vector.

Cell Culture and Inhibitors

Western Blot Analysis

Cells were preincubated overnight with 1 μg/mL of cetuximab or panitumumab and/or 250 nmol/L of JNJ-38877605 in serum-free medium. The day after, they were stimulated with 80 ng/mL of HGF for 15 minutes in the presence of the above-mentioned inhibitors. Cells were lysed in lysis buffer [2% SDS, 0.5 mol/L Tris-HCl (pH 6.8)].  Primary antibodies: anti-actin (1–9), anti-EGFR, and anti-RAS (F234) were from Santa Cruz Biotechnology, anti-MET (clone 3D4) was from Invitrogen; antibodies against phosphorylated MET (Tyr1234/1235), phosphorylated EGFR (Tyr1068), phosphorylated extracellular signal–regulated kinase (ERK; Thr202/Tyr204), phosphorylated AKT (Ser473), total AKT, and ERK were from Cell Signaling. Secondary antibodies were from Amersham. Detection was conducted with enhanced chemiluminescence system (Amersham).

Xenograft Transplantation Experiments

Approximately 5 x 10⁶ tumor cells were injected subcutaneously in 6-week-old immunodeficient nude/beige diabetic/severe combined immunodeficient (NOD/SCID) mice in the presence of 10% Matrigel. Tumor treatment (intratumoral injection of 200 μg of cetuximab twice a week) started when tumor volume reached 200 mm³. The derivation of patient-derived tumor grafts (“xenopatients”) and their profiling for response to treatment with cetuximab was conducted as previously reported (11, 23, 53). Established tumors (average volume,

MET Gene Copy Number by Real-Time PCR

qPCR experiments for estimation of MET copy number variations were conducted in triplicate using a Human TaqMan Copy Number Assay for MET (assay ID: hs01277655_cn) and the TaqMan Copy Number Reference Assay RNase P (Applied Biosystems). Furthermore, to exclude an entire chromosome 7 polysomy, the MADIL1 gene (assay ID: hs00981515_cn), which is located on the p-arm of chromosome 7, was evaluated for Copy Number Variation (CNV). PCR runs were conducted using ABI MicroAmp Optical Fast 96-well Reaction Plates on an Applied Biosystems ViiA 7 Real-Time PCR System (Applied Biosystems) using 20 ng total gDNA as a template. CNV of the target gene was calculated by the ABI SDS software 1.1 using relative quantification based on the ΔΔCt method and control samples as calibrators.

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MET Confers Resistance to EGFR-Targeted Antibodies in Colorectal Cancer

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