Tumor heterogeneity and lesion-specific response to targeted therapy in colorectal cancer

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ABSTRACT

How genomic heterogeneity associated with acquired resistance to targeted agents affects response to subsequent therapy is unknown. We studied EGFR blockade in colorectal cancer to assess whether tissue and liquid biopsies can be integrated with radiological imaging to monitor the impact of individual oncogenic alterations on lesion-specific responses. Biopsy of a patient’s progressing liver metastasis following prolonged response to cetuximab revealed a K57T MEK1 mutation as a novel mechanism of acquired resistance. This lesion regressed upon treatment with panitumumab and the MEK inhibitor trametinib. In ctDNA, mutant MEK1 levels declined with treatment, but a previously unrecognized KRAS Q61H mutation was also identified that increased despite therapy. This same KRAS mutation was later found in a separate non-responding metastasis. In summary, parallel analyses of tumor biopsies and serial ctDNA monitoring show that lesion-specific radiographic responses to subsequent targeted therapies can be driven by distinct resistance mechanisms arising within separate tumor lesions in the same patient.

SIGNIFICANCE

Molecular heterogeneity ensuing from acquired resistance drives lesion-specific responses to subsequent targeted therapies. Analysis of a single-lesion biopsy is inadequate to guide selection of subsequent targeted therapies. ctDNA profiles allow the detection of concomitant resistance mechanisms residing in separate metastases and assessment of the effect of therapies designed to overcome resistance.
INTRODUCTION

Personalized cancer medicine approaches, inhibiting kinases in tumors driven by defined genomic alterations, have demonstrated striking efficacy in many cancer types. However, acquired resistance inevitably develops, limiting the benefit of targeted therapies(1). Acquired resistance mechanisms are typically identified by biopsying a single resistant tumor lesion for molecular analysis. This information is sometimes used to guide subsequent therapy for individual patients. For example, recent trials evaluating therapeutic strategies designed to overcome resistance mechanisms actually require identification of a specific molecular alteration in a post-progression tissue biopsy as a condition for enrollment (NCT02192697, NCT02094261).

However, tumors can display high levels of molecular heterogeneity(2-7). Indeed, exposure to therapy may result in selection of sub-clonal cell populations, capable of growing under drug pressures(8-11). Therefore, a single-lesion biopsy at disease progression may vastly underrepresent the molecular heterogeneity of resistant tumor clones in an individual patient and may fail to detect the existence of distinct but important resistance mechanisms that could impact treatment responses.

The impact of tumor heterogeneity, arising as a result of acquired resistance, on response to subsequent lines of targeted therapy has been hypothesized, but never documented definitively. Here, we show that different metastatic biopsies from the same colorectal cancer (CRC) patient display genetically distinct mechanisms of resistance to EGFR blockade. By assessing multiple biopsies in parallel with circulating tumor DNA (ctDNA) analysis, we demonstrate that distinct resistance mechanisms emerging in different metastases in the same patient can drive lesion-specific responses to the next
RESULTS

Emergence of a MEK1 K57T mutation upon acquired resistance to cetuximab

The patient’s initial clinical course is summarized in Fig. 1A. Following adjuvant chemotherapy for stage IIIc colorectal adenocarcinoma, the patient was found to have a new liver metastasis and tumor recurrence at the site of surgical colonic anastomosis. A simultaneous low anterior resection and partial hepatectomy were performed, but she developed new liver metastases 2 months later.

Molecular analysis of the primary tumor revealed wild-type (WT) KRAS and NRAS genes. The anti-EGFR antibodies, cetuximab and panitumumab, improve survival in combination with chemotherapy in RAS WT CRC(12, 13). The patient responded to palliative chemotherapy with irinotecan and cetuximab for 15 months. The clinical response was attributed to cetuximab, as the patient’s disease progressed while receiving irinotecan-containing chemotherapy as the prior line of therapy. Ultimately, her liver metastases progressed, and a core needle biopsy of a progressing segment 8 liver metastasis was obtained. The patient’s disease continued to progress despite subsequent treatment with FOLFOX and bevacizumab, followed by regorafenib.

Molecular analysis of the post-progression liver metastasis biopsy was performed to determine the mechanism of acquired resistance to cetuximab and to guide subsequent therapy.
The post-progression liver biopsy and the primary tumor were analyzed with a next-generation sequencing panel covering 1000 genes, (Supplementary Table S1). A targeted sequencing panel (Supplementary Table S2) was also performed on these specimens and on two additional tumor specimens obtained prior to treatment with irinotecan and cetuximab (Fig. 1). A truncating mutation in TP53 at codon 171 (p.E171*; c.511g>t) was identified in all tumor specimens, suggesting that this mutation arose early in the clonal development of this CRC (Fig. 1, Supplementary Table S3). A lysine-to-threonine substitution at codon 57 (p.K57T; c.170a>c) of MEK1 (encoded by the MAP2K1 gene) was identified in the post-progression liver lesion, but was not detected in all three tumor specimens obtained prior to cetuximab (Fig. 1, Supplementary Table S3). Mutations in p.K57 in MEK1 were recently implicated in de novo resistance to anti-EGFR antibodies in CRC(14, 15), they have not previously been observed in the setting of acquired resistance. No other alterations previously implicated in resistance to anti-EGFR antibodies(6, 8, 9, 16) were identified, although the presence of additional subclonal resistance alterations not detected in our analysis of this tumor biopsy cannot be ruled out. MEK1 signals downstream of EGFR, and mutations at p.K57 in MEK1 occur in lung adenocarcinoma and can activate MEK1 kinase activity(17, 18). Thus, MEK1 mutation could bypass the effect of EGFR inhibition and likely represents a novel mechanism of acquired resistance to cetuximab in this patient.

**Role of MEK1 mutation in acquired resistance to cetuximab**

Modeling acquired resistance to targeted therapies in cancer cells has proven effective in predicting clinically-relevant resistance mechanisms and in guiding therapeutic strategies to overcome resistance(19, 20). A cetuximab-sensitive RAS-WT CRC cell line (HCA46) was treated with cetuximab until resistant clones emerged. These resistant
clones developed a lysine-to-asparagine substitution at codon 57 (p.K57N) of MEK1—the same codon mutated in the patient’s post-progression biopsy (Fig. 2A, Supplementary Fig. S1A). These cells exhibited constitutive activation (phosphorylation) of MEK and ERK despite cetuximab treatment (Supplementary Fig. S1B). Exogenous expression of either K57T (identified in the patient) or K57N (identified in the cell line) mutant MEK1, but not wild-type MEK1, in an independent RAS-WT CRC cell line, LIM1215, was sufficient to confer resistance to cetuximab or panitumumab (Figure 2B, Supplementary Fig. S1C-D). However, the combination of the MEK inhibitor trametinib with either cetuximab or panitumumab was able to restore sensitivity, confirming that EGFR-dependence is maintained in the setting of acquired resistance, and suggesting a potential therapeutic strategy to overcome resistance to EGFR blockade caused by this mutation (Fig. 2C, Supplementary Fig. S2A-E).

Subsequent targeted therapy and serial ctDNA monitoring

Accordingly, the patient was treated with the combination of panitumumab and trametinib, which have been administered together previously(21). The patient’s serum carcinoembryonic antigen CEACAM5 (CEA) levels decreased by ~60% during therapy (Fig. 3A). A repeat computed tomography (CT) scan of the abdomen after 3 months of therapy demonstrated a reduction in the size of the patient’s segment 8 liver metastasis, which harbored the MEK1 p.K57T mutation (Fig. 3B), but revealed that some other metastatic lesions had in the meantime progressed.

Peripheral blood for plasma ctDNA analysis was collected prior to initiation of panitumumab and trametinib and throughout treatment. Plasma collected prior to therapy was analyzed using a next-generation sequencing method, which we developed
to interrogate 226 cancer-related genes in ctDNA(15). As expected, this analysis detected the TP53 p.E171* and MAP2K1 p.K57T variants, but surprisingly unveiled a previously unrecognized KRAS p.Q61H (c.183a>c) mutation (Supplementary Table S4). Indeed the KRAS p.Q61H mutation was not observed in the segment 8 liver metastasis biopsy by NGS or by high-sensitivity digital droplet polymerase chain reaction (ddPCR) (Fig. 3B, Supplementary Table S3), suggesting that this mutation was not present in this metastasis, but was already present in a separate metastatic lesion at the start of panitumumab and trametinib therapy.

Changes in the relative abundance of specific mutations in ctDNA during panitumumab and trametinib treatment were monitored by ddPCR. Levels of the TP53 p.E171* variant dropped after initiation of therapy, but rose later during treatment in concert with the patient’s CEA levels (Fig. 3A, Supplementary Table S5). Since TP53 p.E171* was detected in all tumor specimens from this patient, it likely represents an early clonal or “founder” mutation present in all tumor cells, and thus a marker of overall disease burden. Another “founder” mutation, IGF1R p.R366W (c.1096c>t), showed a similar pattern (Supplementary Figure S3A-B, Supplementary Tables S5-S6).

However, levels of MAP2K1 p.K57T declined sharply and remained low throughout treatment, indicating effective suppression of MEK1 mutant clones by panitumumab and trametinib. Suppression persisted even as the patient’s CEA and TP53 p.E171* levels began to rise, suggesting that a different molecular alteration must be driving disease progression (Fig. 3A, Supplementary Table S6). Conversely, KRAS p.Q61H rose markedly during therapy, indicating outgrowth of a resistant KRAS-mutant clone. Biopsy of a different segment 5 liver metastasis that progressed despite panitumumab and trametinib revealed that this lesion harbored the same KRAS p.Q61H mutation identified
in ctDNA, along with the TP53 p.E171* mutation, but the MAP2K1 p.K57T mutation was not detected by sequencing or ddPCR (Fig. 3B-C, Supplementary Table S3). Notably, the KRAS or MAP2K1 mutations could not be detected by high-sensitivity ddPCR in any of the tumor specimens obtained prior to the prolonged response to cetuximab (Supplementary Table S3), but preexistence of rare clones harboring these mutations below the limit of detection cannot be excluded.

After 4 months of panitumumab and trametinib, the patient discontinued therapy as CEA levels continued to rise. Analysis of ctDNA obtained one week later revealed a rebound in MAP2K1 p.K57T levels (Fig. 3A).

DISCUSSION

The inevitable emergence of acquired resistance is a major limitation to the efficacy of targeted therapies in oncology. Identification of actionable resistance mechanisms may offer patients the opportunity to benefit from therapies designed to overcome resistance.

Here, we describe how distinct acquired resistance mechanisms can arise concomitantly in separate metastases within the same patient, leading to mixed responses to subsequent targeted therapies. This demonstrates how molecular analysis of a single-lesion biopsy, currently the diagnostic standard for targeted therapy trials, can regularly fail to detect clinically-relevant molecular alterations, which can be responsible for lesion-specific or even sub-clone-specific clinical response and consequent treatment failure.

In this CRC patient, we identified a MEK1 p.K57T mutation in a biopsy of a single
progressing liver metastasis, following prolonged response to cetuximab. Based on preclinical modeling and characterization of this novel resistance mechanism, the patient was treated with the combination of panitumumab and trametinib. Imaging revealed that the lesion harboring the MEK1 mutation responded. However, a neighboring metastasis progressed and was found to harbor a completely distinct resistance mechanism (KRAS p.Q61H), confirming that separate metastases can independently evolve different resistance mechanisms, resulting in striking differences in lesion-specific response to targeted therapy.

Our original single-lesion biopsy was not sufficient to capture the molecular heterogeneity of this patient’s cancer and failed to detect the simultaneous presence of an additional resistance mechanism (KRAS mutation) that eventually led to treatment failure. This underscores the potential pitfalls of selecting a targeted therapy strategy based on the molecular profile of a single resistant lesion. However, both mutations were readily detectable in ctDNA from blood collected prior to combinatorial therapy.

These findings also illustrate the potential of “liquid biopsies”. Not only did real-time ctDNA analysis enable identification of a second resistance mechanism not captured by the single-lesion biopsy, but it did so while the patient still appeared to be responding to therapy, thereby predicting both the timing and cause of impending treatment failure. ctDNA analysis also allowed monitoring of dynamic shifts in the clonal composition of the patient’s tumor cells, demonstrating effective on-target suppression of the MEK1 mutant population by panitumumab and trametinib, contrasted with marked expansion of the KRAS mutant population driving disease progression.
In summary, while it has been proposed that tumor heterogeneity developing in the context of acquired resistance may have the potential to impact response to subsequent therapies, this has yet to be clearly documented. Here, we demonstrate how individual metastatic lesions can develop distinct resistance mechanisms to targeted agents, resulting in lesion-specific differences in response to the next line of targeted therapy. As more trials evaluating targeted therapy strategies designed to overcome specific acquired resistance mechanisms enter the clinic, genomic results from single-tumor biopsies should be interpreted with caution. By contrast, liquid biopsy approaches have the potential to detect the presence of simultaneous resistance mechanisms residing in separate metastases in a single patient and to monitor the effects of subsequent targeted therapies. Therefore, ctDNA profiles, serial tumor biopsies and lesion-specific radiographic responses can be integrated to define mechanisms of drug resistance and to guide selection of therapeutic strategies in oncology.

METHODS

Patient care and specimen collection
All biopsies, tumor specimens, and peripheral blood draws for plasma isolation were collected in accordance with Institutional Review Board-approved protocols, to which patients provided written informed consent, and all studies were conducted in accordance with the Declaration of Helsinki. Targeted exome sequencing on clinical tissue specimens using a Clinical Laboratory Improvement Amendment (CLIA)-certified clinical next-generation sequencing assay was performed in the Department of Molecular Pathology at the Massachusetts General Hospital. The patient was treated with panitumumab and trametinib, both approved by the United States Food and Drug Administration, off-label with informed consent, and the patient's insurance company...
covered the cost of this therapy. Imaging studies, including computed tomography (CT) and Magnetic Resonance Imaging (MRI) scans were obtained as part of routine clinical care.

**Cell lines**

HCA46 CRC cells were obtained from ECACC cell line bank. The LIM1215 parental cell line has been described previously(22) and was a kind gift of Prof. Robert Whitehead, Vanderbilt University, Nashville, with permission from the Ludwig Institute for Cancer Research, Zurich, Switzerland. The genetic identity of cell lines were last authenticated no less than three months before performing experiments by Cell ID™ System and by Gene Print® 10 System (Promega), through Short Tandem Repeats (STR) at 10 different loci (D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, TPOX, CSF1PO and amelogenin). Amplicons from multiplex PCR reactions were separated by capillary electrophoresis (3730 DNA Analyzer, Applied Biosystems) and analyzed using GeneMapperID software from Life Technologies. All cell lines were tested and resulted negative for mycoplasma contamination with Venor GeM Classic Kit (Minerva Biolabs).

**Plasma Sample Collection**

At least 10 mL of whole blood were collected by blood draw using EDTA as anticoagulant. Plasma was separated within 5 hours through 2 different centrifugation steps (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 up to 3 mL of plasma. Plasma was stored at -80°C until ctDNA extraction.

**ctDNA isolation, genome equivalents quantification (GE/ml plasma), and analysis**
ctDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN) according to the manufacturer’s instructions. 6 µl of ctDNA were used as template for each reaction. All samples were analyzed in triplicate. PCR reactions were performed using 10 µl final volume containing 5 µl GoTaq® qPCR Master Mix, 2X with CXR Reference Dye) (Promega) and LINE-1 [12.5 µmol] forward and reverse primers. DNA at known concentrations was also used to build the standard curve. Primer sequences are available upon request. Analysis of ctDNA by NGS and ddPCR was performed as previously described(15). Detailed methods are provided in the Supplementary Methods.

Cell culture and generation of resistant cells
HCA46 cells were cultured in DMEM medium (Invitrogen), supplemented with 10% FBS, 2mM L-glutamine, antibiotics (100U/mL penicillin and 100 mg/mL streptomycin) and grown in a 37°C and 5% CO2 air incubator. LIM1215 were cultured in RPMI medium (Invitrogen), supplemented with 1µg/ml insulin.
HCA46 cetuximab-resistant derivatives were obtained by exposing cells to a chronic dose of 100µg/ml of cetuximab until resistant derivatives emerged.

Mutational analysis in cell lines
Genomic DNA samples were extracted by Wizard® SV Genomic DNA Purification System (Promega). For Sanger Sequencing, all samples were subjected to automated sequencing by ABI PRISM 3730 (Applied Biosystems). Primer sequences for MAP2K1 (exon2) are listed elsewhere(17, 19).

Ectopic expression of MEK1 in CRC cells.
LIM1215 RAS wild-type cetuximab-sensitive cells were cultured in RPMI medium (Invitrogen) supplemented with 10% FBS, 1µg/ml insulin, 2mM L-glutamine, antibiotics (100U/mL penicillin and 100 mg/mL streptomycin) and grown in a 37°C and 5% CO2 air incubator. LIM1215 were transduced with lentiviral vector encoding MEK1 WT, MEK1 K57N or MEK1 K57T cDNA. MEK overexpression was verified by western blot analysis.

**Drug proliferation assay**

CRC cell lines were seeded at different densities (2-3 x10³ cells/well) in 100µl complete growth medium in 96-well plastic culture plates at day 0. The following day, serial dilutions of the indicated drugs were added to the cells in serum-free medium, while medium-only (in case of cetuximab and panitumumab) or DMSO-only (in case of trametinib) treated cells were included as controls. Plates were incubated at 37°C in 5% CO2 for 4 or 5 days, after which cell viability was assessed by measuring ATP content through Cell Titer-Glo® Luminescent Cell Viability assay (Promega). Luminescence was measured by Perkin Elmer Victor X4.

**Western blotting analysis**

Prior to biochemical analysis, all cells were grown in their specific media supplemented with 10% FBS with or without indicated drug treatment. Total cellular proteins were extracted by solubilizing the cells in EB buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5mM EDTA, 2mM 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. Extracts were clarified by centrifugation, normalized with the 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-MEK1/2 (Ser217/221), anti-MEK1/2; anti-phospho AKT (T308); anti-AKT; anti-vinculin (Millipore).

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FIGURE LEGENDS

**Figure 1: Initial treatment course and analysis of serial tumor biopsies.**

The initial clinical course of the CRC patient is summarized, with serum carcinoembryonic antigen (CEA, normal defined as <3.5 ng/mL) tumor marker levels shown throughout treatment. Shaded areas indicate periods of administration of the indicated chemotherapeutic agents: 5-fluorouracil (5FU), oxaliplatin (OX), irinotecan (IRI), cetuximab (CET), radiation therapy (XRT), bevacizumab (BEV), regorafenib (REG). Arrows indicate timing of tumor specimen acquisition from surgical procedures or biopsy. At the bottom of panel A, sequencing data for each specimen are summarized. A p.K57T missense mutation in the *MAP2K1* gene (which encodes for MEK1 protein) was detected in a progressing liver lesion following a prolonged response to cetuximab and irinotecan. The *MAP2K1* p.K57T mutation was not detected in tumor specimens gathered prior to cetuximab and irinotecan therapy (specimens 1-3). Conversely, a nonsense mutation in *TP53* at codon 171 (p.E171*) was detectable in all tumor specimens throughout the clinical course. Variant reads as a fraction of total sequencing reads are shown, with the variant allele percentage shown in parentheses.

**Figure 2: MEK1 K57 mutations confer resistance to anti-EGFR therapies.**

(A-C) Cetuximab-resistant preclinical models were derived *in vitro* from HCA46, a RAS-wildtype CRC cell line. (A) The sensitivity of parental cells and resistant clones to cetuximab and panitumumab in an *in vitro* viability assay are shown. (B) Exogenous expression of MEK1 K57T or MEK1 K57N in an independent cetuximab-sensitive RAS-WT CRC cell line (LIM1215) confers resistance to cetuximab, relative to expression of WT MEK1. (C) The combination of 50µg/ml cetuximab (Cetux) and 2nM trametinib (Tramet) or 50µg/ml panitumumab (Panit) and 2nM trametinib can restore sensitivity to
MEK1 mutated cetuximab-resistant clones.

**Figure 3. Serial analysis of plasma circulating tumor DNA during subsequent therapy with panitumumab and trametinib.**

(A) Timing of panitumumab and trametinib administration is denoted by the grey bar. Timing of discontinuation of therapy is indicated by the dashed line. Serum CEA levels were monitored throughout treatment. Serial assessments of plasma circulating tumor DNA for the percent abundance of variant alleles for *TP53* p.E171* (an early mutational event, present in all of the patient’s tumor cells), *MAP2K1* (MEK1) p.K57T, and *KRAS* p.Q61H are shown throughout treatment. While levels of the known *MAP2K1* p.K57T mutation decreased during therapy with panitumumab and trametinib, a *KRAS* p.Q61H mutation was discovered in the plasma, which increased steadily throughout treatment. (B) Axial CT images of the abdomen taken at the start of panitumumab and trametinib therapy (July 2014) and after approximately three months of therapy (November 2014) show a decrease in the size of the segment 8 liver lesion, which harbored the MEK1 K57T mutation. Sequencing data from the biopsy of this lesion obtained after progression on cetuximab and irinotecan and prior to panitumumab and trametinib therapy is summarized on the right. Variant reads as a fraction of total reads are shown, with the variant allele percentage shown in parentheses. (C) Conversely, CT images show that a segment 5 liver lesion increased in size despite therapy with panitumumab and trametinib over the same time period. The segment 5 liver lesion was biopsied after progression on panitumumab and trametinib, and next-generation sequencing detected the same *KRAS* p.Q61H mutation that was identified in the plasma ctDNA, as well as the *TP53* p.E171* mutation present in all tumor specimens from this patient, as summarized on the right. The *MAP2K1* p.K57T mutation that was present in the segment 8 liver lesion was not detected in this biopsy of the segment 5 liver lesion, suggesting
independent evolution of distinct resistance mechanisms in these two metastatic lesions.
variant | 1.) Left colectomy | 2.) Low anterior resection | 3.) Partial hepatectomy | 4.) Post-progression biopsy (liver lesion)
--- | --- | --- | --- | ---
*TP53* p.E171* | 124/363 (34.2%) | 5/93 (5.4%) | 167/322 (51.9%) | 299/474 (63.1%)
*MAP2K1* (MEK1) p.K57T | 0/93 (0%) | 0/89 (0%) | 0/416 (0%) | 35/309 (11.3%)
A

- HCA46 Parental_cetuximab
- HCA46 Parental_panitumumab
- HCA46 Resistant_cetuximab
- HCA46 Resistant_panitumumab

% Cell viability (relative to control)

Log [M] Drug

B

- LIM1215 NT
- LIM1215 MEK1 WT
- LIM1215 MEK1 K57N
- LIM1215 MEK1 K57T

% Cell viability (relative to control)

Log [M] cetuximab

C

HCA46 Resistant

% Cell viability (fold control)

tramet  +  -  +  -  +
panit  -  +  +  -  -
cetux  -  -  -  +  +

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Panitumumab + trametinib

**A**

- CEA (ng/mL)
- TP53 p.E171*

**B**

- Segment 8 liver lesion
- Segment 5 liver lesion

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**C**

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Russo et al, Fig. 3
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