Resistance to Anti-EGFR Therapy in Colorectal Cancer: From Heterogeneity to Convergent Evolution

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The EGFR-targeted antibodies cetuximab and panitumumab are used to treat metastatic colorectal cancers. Mutations in KRAS, NRAS, and BRAF and amplification of ERBB2 and MET drive primary (de novo) resistance to anti-EGFR treatment. Recently, the emergence of alterations in the same genes was detected in patients who responded to EGFR blockade and then relapsed. These results illuminate a striking overlap between genes that, when mutated, drive primary and secondary resistance to anti-EGFR antibodies. Remarkably, although the mechanisms of resistance are genetically heterogeneous, they biochemically converge on key signaling pathways. This knowledge is being translated in the rational design of additional lines of therapy.

Significance: Anti–EGFR-targeted therapies are used for the treatment of metastatic colorectal cancer. Molecular heterogeneity impairs their efficacy by fuelling de novo and acquired resistance. In this review, we highlight how genetically distinct resistance mechanisms biochemically converge on a limited number of signaling pathways that can be therapeutically intercepted.

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THE HETEROGENEOUS MOLECULAR LANDSCAPE OF COLORECTAL CANCER

On December 27, 1831, Charles Darwin left Plymouth Harbor on board the H.M.S. Beagle to begin a long journey that, more than any other, transformed scientific knowledge. While traveling, Darwin had the opportunity to observe and collect samples from heterogeneous animal and vegetal species on the landscape of multiple continents. His rigorous scientific method established the basis of the unifying theory of life sciences, ultimately explaining the diversity of life.

Planet Earth hosts ecosystems endowed with extraordinarily diverse environmental conditions; these conditions exert selective pressures, which enable evolution. Comparable selective pressures foster the development of parallel evolutionary results in unrelated species living in distinct ecosystems (convergent evolution). A classic example of this phenomenon is the convergent evolution of wings or fins in birds and mammals.

In addition to his exceptional intuitive thinking, Darwin was able to “quantify” his observations by visiting several locations and analyzing the results of evolutionary processes in all of these environments.

In 1976, Peter Nowell stated that “tumor progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines,” and, most importantly, that “more research should be directed toward understanding and controlling the evolutionary process in tumors before it reaches the late stage usually seen in clinical cancer” (1).

Each individual tumor can be seen as a microcosm under incessant variation based on genetic diversity (heterogeneity), selection, and evolution: the very same mainstays on which life is based, although tumors proceed through them at a much faster pace. Just as Darwin did more than a century ago with the complexity of speciation, research in oncology strives to understand the intricacy of cancer.

The ability to explore (which we define as the ability to molecularly annotate) cancer genomes can be seen as a modern version of the H.M.S. Beagle. By applying next-generation sequencing (NGS) technologies to scan the cancer genome, the oncology community has recently (re)discovered molecular heterogeneity in tumor samples (2), including those of colorectal origin (3).

Colorectal cancer, the third most common cancer type in Western countries, affects more than 200,000 patients worldwide every year (4). Screening, surgery, and medical therapies are successful in the management of early-stage colorectal...
cancer, but far less efficacious in advanced stages of the disease. A key reason for the limited success of colorectal cancer-directed therapies is the cancer’s intrinsic heterogeneity, which is more prominent in the metastatic setting (5, 6). Molecular characterization of colorectal cancers revealed that heterogeneity plays an important role, especially in the context of resistance to therapy. More than half of colorectal cancers display heterogeneous genetic alterations in genes involved in EGFR signaling, which negatively affect response to the monoclonal antibodies cetuximab and panitumumab. Molecular heterogeneity has been recognized as pivotal in the evolution of clonal populations during anti-EGFR therapies. In this review, we provide an outline of how genetic diversity (molecular heterogeneity) influences primary (de novo) and secondary (acquired) resistance to EGFR-targeted therapies in colorectal cancer.

**MECHANISMS OF PRIMARY RESISTANCE TO EGFR-TARGETED THERAPY IN COLORECTAL CANCER**

**Known Culprits**

The EGFR-directed monoclonal antibodies cetuximab and panitumumab were approved to treat patients with chemorefractory metastatic colorectal cancer (mCRC) in 2004 and 2006, respectively (Fig. 1). Both drugs have very similar efficacy, achieving objective response rates of approximately 10% when used as monotherapy for irinotecan-refractory and/or oxaliplatin-refractory mCRC (7, 8). Investigations into the molecular basis of response to EGFR-blocking antibodies started in 2005 and were based on retrospective analyses of archived tumor tissue from subsets of patients participating in clinical trials (9). Since then, a rapidly accumulating body of knowledge has indicated that resistance to EGFR blockade in mCRC is related to constitutive activation of signaling pathways downstream of EGFR. Mutations in KRAS occurring at codons 12 and 13 were the first to be causally implicated in resistance to EGFR-targeted monoclonal antibodies, initially in small patient cohorts (10, 11). Randomized phase III studies provided compelling evidence that led regulatory authorities to exclude patients with chemorefractory mCRC with tumors bearing KRAS mutations from treatment with single-agent cetuximab or panitumumab (12, 13). In 2009, the analysis of KRAS codon 12 and 13 mutations as a test to restrict the use of cetuximab in combination with chemotherapy to first-line mCRC patients with wild-type tumors gained regulatory approval (14, 15).

Because not all KRAS wild-type patients benefit from treatment with EGFR-directed therapy, research has flourished to
identify additional biomarkers of resistance that could account for the heterogeneity in clinical response. Sequencing studies revealed that although more than 80% of \(KRAS\) variants occur in exon 2 at codons 12 and 13, oncogenic mutations also affect \(KRAS\) codons 59, 61, 117, and 146 (16–18). Additional mutations of the \(NRAS\) isoform occur at codons 12, 13, and 61 in approximately 3% to 5% of colorectal cancer samples (19). Figure 2 summarizes the incidence of \(RAS\) mutations in exon 2 (including codons 12 and 13), exon 3 (comprising codons 59 and 61), and exon 4 (which includes codons 117 and 146). Mutations in \(KRAS\) or \(NRAS\) lead to continuous activation of downstream ERK signaling, regardless of whether the EGFR is pharmacologically inactivated. Although the role of the canonical exon 2 mutations is considered uncontroversial, the exact properties of the less-frequent mutations have not been fully elucidated. However, data from retrospective studies indicate that \(RAS\) mutations occurring beyond \(KRAS\) exon 2 could also underlie lack of response to single-agent cetuximab or panitumumab in patients with chemorefractory mCRC (20–23). Multiple studies have recently shown that mutations in \(KRAS\) exons 3 and 4 or \(NRAS\) exons 2 to 4 can also predict lack of clinical benefit to EGFR-targeted antibodies given in combination with first-line chemotherapy (24–26).

**Under Scrutiny**

When combined, \(RAS\) and \(BRAF\) mutations account for more than 60% of patients with mCRC who show de novo resistance to EGFR-targeted monoclonal antibodies. Beyond \(RAS\) and \(BRAF\) point mutations, numerous genetic alterations in genes implicated in EGFR signaling play a role in de novo resistance. Importantly, although molecularly heterogeneous, these alterations biochemically converge on activation of the RAS–MEK–ERK pathway.
KRAS gene amplification occurs in 1% to 2% of colorectal cancer cases and has been reported to be nearly always mutually exclusive with KRAS mutations (18, 32, 33). KRAS gene amplification has been shown to cause resistance to cetuximab in functional genetics experiments and has been associated with lack of response to anti-EGFR treatment (32, 33). Given the low prevalence of KRAS gene amplification, its association with refractoriness to EGFR blockade did not reach statistical significance. An analysis from the TCGA colorectal cancer database (34) has revealed that gene amplification can also occur in NRAS, BRAF, and CRAF at a very low prevalence (<1% cases for individual genes), but the clinical relevance of these findings is unknown.

Additional genetic mechanisms have been proposed to activate the EGFR-RAS pathway in the absence of molecular alterations affecting RAS or its immediate downstream effectors. Genetic aberrations of the receptor tyrosine kinases (RTK) ERBB2 and MET have been shown to bypass EGFR signaling and activate the MEK-ERK cascade. ERBB2 gene amplification was found in a small fraction of RAS and BRAF wild-type mCRC patient-derived xenografts that were insensitive to cetuximab treatment. These results were corroborated by the identification of ERBB2 amplification in samples from patients with mCRC who did not benefit from EGFR-targeted treatment (29). Concordant data were obtained by Yonesaka and colleagues (35), who showed that activation of ERBB2 signaling, dependent on either gene amplification or overproduction of the ERBB3 ligand heregulin, was present in a subset of patients with mCRC exhibiting de novo resistance to cetuximab-based therapy. Another kinase receptor, MET, is amplified in a small fraction (2%) of mCRC samples unsel ected for their sensitivity to anti-EGFR therapy (34, 36–38). Once again, amplified MET was found in a small fraction of RAS and BRAF wild-type mCRC patient-derived xenografts that were insensitive to cetuximab treatment (38). Therefore, these pathways may offer primary “escape mechanisms,” allowing tumors to circumvent one pathway that has been pharmacologically blocked.

Bystanders or Partners in Crime?

The overall scenario is further complicated by the existence of additional colorectal cancer genetic alterations in EGFR signaling that might confer resistance to cetuximab or panitumumab. For example, the PI3K-AKT-PTEN pathway can also be triggered by EGFR activation; therefore, several studies were conducted to define whether molecular alterations of these genes could also impair response to EGFR-targeted monoclonal antibodies. Results obtained by multiple laboratories associate PIK3CA exon 20 mutations with unresponsiveness to anti-EGFR monoclonal antibodies; however, the correlation is not strong enough to be applied as a clinically valuable negative predictive marker of response, possibly due to the relatively small sample size of each study and the confounding effect of concomitant chemotherapy administration (20, 39–48). PTEN status is also associated with a lack of response, but also in this case, results remain inconclusive, partially because of difficulties in assessing the status of PTEN in clinical specimens (30, 40, 42, 49–56). Moreover, PIK3CA and PTEN alterations (around 10%–15% overall) often co-occur with KRAS or BRAF mutations (20, 30, 34, 44), a feature that further complicates their assessment. In summary, the role of PIK3CA mutation and PTEN status in conferring resistance to EGFR-directed therapy in colorectal cancer remains highly controversial.

Other Suspects

The genetic mechanisms described above do not account for the totality of patients who show clinical resistance to anti-EGFR drugs. Indeed, for approximately 10% of cases, the genetic alteration that confers de novo resistance is presently unknown. We hypothesize that when a patient fails to respond to anti-EGFR treatment, the most likely cause is the occurrence of a yet-to-be-reported genetic alteration in either an RTK, a downstream amplifier of the RTK-initiated signal, or a key node of the EGFR signaling pathway.

Most likely, these will be found in genetic alterations in known oncogenes, such as amplification or translocations of RTK genes identified by the TCGA in colorectal cancer samples that do not harbor RAS or BRAF mutations, such as NTRK, RET, ALK, or ROS1 (34). These additional oncogenic events are present at low prevalence (<1%–5%), and analyses of large datasets will be required for their clinical validation.

Alternatively, it is possible that well-known alleles (such as RAS mutations) are present in the tumor at a prevalence that cannot be detected by commonly used techniques. The low-sensitivity issue has its roots in tumor heterogeneity. Tissue biopsies represent a small fraction of the entire tumor burden. This assumption means that, because of intratumor and/or intermetastases heterogeneity, analysis of tissue from an individual biopsy may not capture its entire molecular complexity. The analysis of multiple biopsies from a single patient revealed the presence of several subclones that may be present or absent in different metastases or the primary site. Furthermore, the same single lesion can harbor more than one independent clone (2, 57, 58). These observations are particularly relevant when considering that previous studies mainly involved analysis of KRAS exon 2 mutations, and that the most commonly used techniques (Sanger sequencing) have a limit of detection of approximately 15% to 20% (59). Of interest, it has been shown that more sensitive approaches such as pyrosequencing or digital PCR can increase the detection of mutant RAS alleles, which in turn could translate into the detection of additional refractory patients (22, 57, 58, 60, 61).

Finally, although in this report we focused mainly on genetic heterogeneity as a basis for the complexity observed in resistance to EGFR inhibition in colorectal cancer, non-genetic mechanisms could also play a role in resistance to EGFR blockade (and are definitely relevant with other targeted agents in different cancers). Intriguingly, in biopsies from patients who relapsed upon cetuximab or panitumumab therapy, only a fraction of cells carry RAS mutations, suggesting that wild-type cells can also survive the treatment (62). This finding suggests that nongenetic mechanisms could also play a role in driving acquired resistance to EGFR blockade. For example, a recent report (63) shows that sensitive (wild-type) cells can survive in the presence of cetuximab when in the company of their resistant derivatives. Notably, it was found that cells bearing acquired RAS mutations over-secrete the EGFR ligands TGFα and amphiregulin, which protect the surrounding wild-type cells (63).
network could potentially be targeted to increase the efficacy of anti-EGFR therapies.

**What Drives Sensitivity to EGFR Blockade in Colorectal Cancer? The EGFR Ligands Hypothesis**

The molecular basis underlying response to EGFR-targeted therapies in colorectal cancer remains obscure. Several studies showed that increased *EGFR* gene copy number correlates with response to cetuximab or panitumumab, in preclinical models and in retrospective clinical analyses (9, 29, 30, 64, 65–67). Nevertheless, this alteration is not currently used as a predictive biomarker because of the difficulties in interlaboratory reproducibility of the diagnostic assay (68).

Although the molecular bases of sensitivity to EGFR blockade are unclear, the clinical efficacy of EGFR-targeted monoclonal antibodies provides evidence that EGFR signaling plays a prominent role in certain colorectal cancers. We propose that dependency on EGFR ligands (via a paracrine–juxtacrine network) is the main oncogenic driver in the colorectal cancers that display sensitivity to cetuximab and panitumumab. In these tumors, activation of the EGFR RAS–MEK axis is not sustained by mutations of downstream effectors, but rather may be achieved by the overproduction of EGFR ligands. Classic studies on viral oncogenes led to the identification of EGFR ligands as being equally effective in triggering cell transformation as RAS. In these colorectal tumors, anti-EGFR antibodies may act by interfering with ligand-dependent activation of EGFR, leading to downregulation of the receptor from the cell surface (69, 70).

**MECHANISMS OF SECONDARY RESISTANCE TO ANTI-EGFR THERAPY IN COLORECTAL CANCER**

**Mutations of the EGFR Extracellular Domain**

In a subset of patients with colorectal cancer, the addition of anti-EGFR monoclonal antibodies to the conventional chemotherapeutic regimens expands response rates, increases progression-free survival, and improves the quality of life. However, the duration of this response is only transient and does not last more than 3 to 12 months, after which secondary resistance occurs. Several studies based on preclinical models and tumor samples obtained at relapse identified molecular mechanisms that lead to acquired resistance to EGFR blockade in colorectal cancer.

Montagut and colleagues (71) discovered a point mutation in the extracellular domain of EGFR (S492R) in a colorectal cancer cell line made resistant to cetuximab. This mutation impairs binding of the antibody to the receptor and was also found in very few patients at relapse after cetuximab treatment. The S492R mutation does not interfere with the binding of panitumumab. Thus, patients with tumors showing the S492R mutation at relapse could be, in principle, treated with panitumumab. Indeed, they reported that a patient harboring the S492R allele as a mechanism of secondary resistance to cetuximab was subsequently treated with panitumumab and responded transiently to this therapy. Notably, the crystal structure of cetuximab bound to the extracellular domain of the EGFR indicates that S492R likely interferes with ligand binding (72). Because other residues in the extra-

**Amplification of RTKs**

Amplification of genes encoding for RTKs is also associated with secondary resistance to anti-EGFR monoclonal antibodies. *ERBB2* or *MET* gene amplifications were described as drivers of acquired resistance to EGFR blockade in cell models and patient samples (35, 38). Several reports confirmed the initial results on the emergence of *MET* gene amplification in patients who develop acquired resistance to EGFR blockade (73, 74).

**Mutations in RAS Genes**

The most common molecular mechanisms that drive secondary resistance to anti-EGFR therapy in colorectal cancer are genetic alterations of the *KRAS* gene (both point mutations and gene amplification). The emergence of *NRAS* and *BRAF* mutations is likewise associated with secondary resistance (62, 75–77).

Of note, *KRAS*, *NRAS*, and *BRAF* mutations, as well as amplification of the *MET* or *ERBB2* genes, are also key drivers of primary resistance to anti-EGFR antibodies in colorectal cancer. Remarkably, although the genetic drivers of primary resistance are usually homogeneous within an individual tumor, more than one driver alteration can emerge in a single tumor at relapse.

Colorectal cancer cell lines made resistant to cetuximab or panitumumab showed the concomitant presence of diverse genetic mechanisms; for instance, in one single resistant cell model, we were able to identify multiple *KRAS* mutations, together with *NRAS*-mutant clones as well (76). The genetic landscapes of cell models are generally considered molecularly homogeneous; however, these experiments suggest that the resistant population may arise upon the selection of multiple clones that were presumably already present at the beginning of the treatment.

The intrinsic genetic heterogeneity that sustains acquired resistance to anti-EGFR antibodies in preclinical models was confirmed in clinical samples from patients with colorectal cancer at relapse after anti-EGFR treatment. Bettegowda and colleagues (77) analyzed circulating cell-free tumor DNA obtained from plasma samples of patients with colorectal cancer at relapse with ultrasensitive technologies. Seventy-six genetic alterations were detected at resistance, all of which were absent in samples from the same patients at the beginning of the treatment. Half of the alterations were in *KRAS* codons 12 or 13; mutations in *BRAF* (V600E) were observed in 2 patients. Interestingly, in 2 patients, mutations in the kinase domain of *EGFR* (codons 714 and 794) were identified. These genetic alterations were not previously described as a mechanism of *de novo* or acquired resistance. Consequently, further studies are needed to understand whether these mutations can confer resistance to anti-EGFR therapy.

Altogether, these results demonstrate that heterogeneity is a feature of resistance to anti-EGFR therapy in colorectal cancer and that intratumor molecular complexity is even
more evident in the context of acquired resistance (Fig. 3 and Table 1). We postulate that the effect of pharmacologic treatment represents a selective pressure, which allows the selection of (preexisting) subclones that confer resistance to the drug. If this is the case, a number of questions arise. Is the presence of the resistant alleles a completely stochastic process? Or does a tumor maintain a reservoir of these subclones? Furthermore, why were these mutations not selected before the drug pressure, similar to those that confer primary resistance? It is conceivable that subclones that emerge after the drug treatment are less fit in the untreated tumor and acquire fitness as a consequence of adaptation to the perturbation induced by the treatment itself. This event has been previously shown to occur in other cancer types. Chmielecki and colleagues (78) demonstrated that erlotinib-resistant NSCLC cells grew more slowly than their sensitive counterparts, and, interestingly, resistance was not maintained in the absence of the drug. A similar phenomenon has been described for BRAF-mutant melanoma cells, which become resistant to vemurafenib through expression of EGFR (79).

These data also highlight the importance of the use of high-sensitivity sequencing technologies for the detection of mutant alleles in colorectal cancer samples. A considerable fraction of patients who are eligible for anti-EGFR treatment develop secondary resistance in a very short time frame. This could be explained as a higher frequency of preexisting resistant clones in the initial population, which cannot be detected by standard sequencing but could be found with more sensitive technologies.

The overall compendium of molecular mechanisms driving acquired resistance to cetuximab and panitumumab is likely incomplete. Although the role of RAS mutations and MET gene amplification in conferring acquired resistance to EGFR blockade has been confirmed by several studies both in preclinical models and in patients (62, 73–77), candidate gene analysis does not always explain the mechanism by which a colorectal cancer becomes resistant to anti-EGFR therapy. Accordingly, further studies will likely characterize additional oncogenic alterations involved in acquired resistance to cetuximab and panitumumab in colorectal cancers. Importantly, results from both cell models and clinical specimens indicate that every patient and, possibly, every metastatic lesion will develop several independent mechanisms of resistance to EGFR blockade (38, 74, 76). It is therefore unlikely that we could obtain a complete profile of the molecular changes occurring in each metastatic patient who becomes resistant.

Figure 3. Molecular heterogeneity drives secondary resistance to anti-EGFR therapies in mCRC. Response to anti-EGFR targeted therapies in mCRC is accompanied by selection of preexisting resistant clones present in the initial metastasis burden. Conceivably, resistant clones can also emerge during treatment. Clones carrying distinct molecular alterations such as KRAS, NRAS, EGFR, and BRAF mutations or KRAS, HER2, or MET amplifications can coexist in the same metastatic site or in different metastatic sites. CT scans were obtained from a colorectal cancer patient who showed the first response to cetuximab observed at Ospedale Niguarda Ca’ Granda in 2001.
Table 1. Summary of genetic alterations associated with secondary resistance to EGFR blockade in mCRCs

<table>
<thead>
<tr>
<th>Reference/study</th>
<th>Genetic alterations at secondary resistance</th>
<th>Tumor sample type</th>
<th>Number of patients</th>
<th>Number of patients displaying more than one genetic alteration at onset of resistance</th>
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<tr>
<td>Yonesaka et al. [35]</td>
<td>HER2 amplification</td>
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<td>Montagut et al. [71]</td>
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<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td>BRAF mutations</td>
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<td>Diaz et al. [75]</td>
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<td>Plasma</td>
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<td>3/24</td>
</tr>
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<tr>
<td></td>
<td>KRAS amplification</td>
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<tr>
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<tr>
<td></td>
<td>KRAS mutations</td>
<td>Plasma and tissue</td>
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<tr>
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<td></td>
<td>MET amplification</td>
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**PRIMARY AND ACQUIRED RESISTANCE TO EGFR BLOCKADE: WHAT IS THE DIFFERENCE?**

**The Primary = Secondary Rule**

EGFR-targeted therapies are commonly used in the treatment of different tumor types of epithelial origin, including NSCLC and colorectal cancer (80). Although the role of EGFR in the pathogenesis of these two cancers is distinct (in NSCLC, EGFR is activated by mutations, whereas in colorectal cancer, it is stimulated by ligands), interesting observations can be made by comparing these two malignancies.

The mechanisms of acquired resistance to anti-EGFR antibodies in colorectal cancer can be broadly categorized in three groups (Fig. 4). The first mechanism includes mutations that disrupt binding of cetuximab (or panitumumab) to the EGFR. This mechanism is analogous to the T790M mutation seen in untreated colorectal cancer (86) and is apparently found only in colorectal cancer samples from patients who have been previously exposed to cetuximab. This is consistent with the hypothesis that this allele evolves as cells strive to evade the selective pressure exerted by the environment during cancer progression. It is conceivable that the pressure that selects for KRAS, NRAS, or BRAF mutations must act in a similar manner in both settings. What are these pressures? We speculate that during the transition from adenoma to carcinoma (that is, when RAS mutation events are thought to occur in the colorectal tumorigenesis sequence), a sudden lack of EGFR activation triggers the outgrowth of clones that are EGFR independent, but are still dependent on its downstream signaling. Indeed, it is known that intestinal epithelial cells depend upon EGFR ligands (85). We speculate that a sudden loss in the availability of EGFR ligands during the adenoma–carcinoma sequence selects for cancerous cells carrying RAS mutations. In a few instances, colorectal cancer cells overcome this pressure not by acquiring downstream pathway mutations but by gaining the ability to self-produce the EGFR ligands needed to sustain pathway activation. Such tumors maintain dependency/sensitivity to EGFR blockade in the later stages of colorectal cancer progression and define the subset of patients that obtain a clinical benefit from cetuximab and panitumumab.

**The Primary = Secondary Rule Has Exceptions**

The EGFR extracelluar domain mutation S492R represents the most notable exception to the primary = secondary rule. The S492R allele has never been detected to date in untreated colorectal cancer (86) and is apparently found only in colorectal cancer samples from patients who have been previously exposed to cetuximab. This is consistent with the hypothesis that this allele evolves as cells strive to evade the EGFR blockade imposed by the monoclonal antibody cetuximab, and accordingly remain sensitive to panitumumab, which binds to a different EGFR epitope located on the extracellular domain of EGFR.
Even more intriguing is the other exception to the primary acquired rule. Remarkably, the relative frequency of individual KRAS alleles is similar but not identical in primary and acquired resistance. For instance, mutations of codon 61 in either the KRAS or NRAS genes are more prevalent in the acquired than in the primary resistance setting (77). This suggests that the selective pressure that results in the acquisition of KRAS mutations during the transition from adenoma to carcinoma is again similar but not identical to the one applied by EGFR blockade (Fig. 4).

**GENETIC HETEROGENEITY AND BIOCHEMICAL CONVERGENCE**

**All Roads Lead to Rome**

In colorectal tumors that respond and then relapse after anti-EGFR treatment, several genetic alterations concomitantly emerge. This phenomenon is best observed by analyzing circulating free DNA from patients at relapse (62, 74–77), which offers a wide-angle perspective of the overall heterogeneity of the disease. This indicates that drug treatment triggers the evolution of multiple subclones, each carrying distinct genetic alterations. Not unlike classic Darwinian evolution, the concomitant presence of several escape mechanisms reflects the high level of molecular heterogeneity present in each metastatic site, which enables the evolutionary processes.

The evolution of secondary resistance to anti-EGFR therapy can be defined as the consequence of a perturbation in a system in which the initial equilibrium is based on cells that are highly dependent on EGFR signaling. The finding that most of the mutations that emerge upon treatment involve genes that are direct members of the EGFR pathway (EGFR, KRAS, NRAS, or BRAF) indicates that to escape the perturbation, the cells must settle on a new balance, which is (has to be) again based on a certain level of EGFR signaling output.

This hypothesis is supported by a biochemical analysis of cell models of colorectal cancer that developed resistance to EGFR blockade regardless of the gene/mutation that confers resistance; the net output was always sustained activation of MEK and ERK, thus defining an example of convergent evolution (76).
Convergent evolution occurs when different species that are phylogenetically unrelated, but placed in the same kind of environment or stimuli, develop parallel morphologic features. A classic example of convergent evolution was observed when unrelated species of mammals, reptiles, and birds evolved “mechanical” features (wings) to be able to fly. Analogously, we postulate that when EGFR blockade occurs in a patient with colorectal cancer with multiple metastatic lesions, the drug pressure triggers the convergent biochemical evolution of independent clones, each of which reactivates the EGFR signaling output. Accordingly, although individual metastases develop what appear to be genetically heterogeneous resistance mechanisms, these are in fact highly related, as they are aimed at reactivating the EGFR signaling pathway at the biochemical level. These findings have several implications that are discussed in the next paragraph.

CONCLUSIONS
Exploiting the Knowledge: The Preemptive Strike Hypothesis
The awareness that solid tumors, which initially respond and then relapse to a targeted therapy, will eventually become highly molecularly heterogeneous poses a formidable therapeutic challenge. At first glance, it would seem arduous to overcome the multiple resistance mutations that arise in each individual patient. Although the overall picture is looming and complex, this knowledge offers several opportunities that may be therapeutically exploited. For example, in patients with colorectal cancer who receive anti-EGFR therapies, the plethora of alterations that emerge at relapse can be intercepted by interfering downstream in the signaling nodes that we now know are aimed at reactivating the EGFR signaling pathway (without offering the tumor the possibility to first escape the probable resistance pathway output). This knowledge can be exploited in several ways. First, it suggests that at relapse, the distinct resistance mechanisms can be intercepted by interfering downstream in the pathway where the signal outputs generated by the distinct genetic events converge, in this case at the MAPK–ERK level. A second and possibly even more relevant implication is that it may be more challenging for a colorectal tumor to escape the EGFR blockade if the initial treatment is designed to concomitantly block the signaling nodes that we now know provide an escape (resistance) route. We hypothesize that if the most probable escape route is blocked from the beginning (without offering the tumor the possibility to first escape the initial treatment), the time required to develop resistance will be extended. In this regard, it will be important to assess, initially in preclinical models, whether the time it takes for colorectal cancer cells to develop resistance to EGFR blockade is extended significantly when the probable resistance pathway output (MEK reactivation) is concomitantly tackled. We postulate that if this scenario is confirmed, this theory will provide unique opportunities for the design of innovative clinical trials that will not await the inevitable development of resistant clones, but rather will attempt their preemptive suppression.

Disclosure of Potential Conflicts of Interest
A. Sartore-Bianchi is a consultant/advisory board member for Amgen and Bayer. S. Siena reports receiving a commercial research grant from Bayer and is a consultant/advisory board member for AstraZeneca, Roche, Sanofi-Aventis, Ignyta, Bayer, and Merck. No potential conflicts of interest were disclosed by the other authors.

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