Chapter 1
Resistance of Colorectal Tumors to Anti-EGFR Antibodies

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Abstract Only a small fraction (10%) of genetically unselected patients with chemorefractory metastatic colorectal cancer benefits from the anti-EGFR antibodies cetuximab or panitumumab ('primary' or 'de novo' resistance). Further, almost all patients who initially respond become resistant over the course of treatment ('secondary' or 'acquired' resistance). Studies in cell lines, patient-derived tumorgrafts, and archival surgical specimens have identified many biomarkers of both primary and acquired resistance to anti-EGFR antibodies, and it is now evident that resistance mechanisms revolve around common genetic lesions and share analogous signaling traits. Here we discuss how resistance to the EGFR blockade is attained in colorectal cancer and elaborate on alternative therapeutic strategies that are now under development to improve response and contrast relapse.

Keywords Colorectal cancer • Epidermal growth factor receptor • Mitogen-activated protein kinase kinase • Drug resistance

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Abbreviations

BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CRC	Colorectal cancer
ctDNA	Circulating tumor DNA
EGFR/ErbB1/HER1	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
HER2/neu/ERBB2	V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
mCRC	Metastatic colorectal cancer
MEK	Mitogen-activated protein kinase kinase
moAbs	Monoclonal Antibodies
NRAS	Neuroblastoma RAS viral oncogene homolog
PIK3CA	Phosphatidylinositol 3-kinase, catalytic, alpha
PTEN	Phosphatase and tensin homolog
RR	Response rate
RTKs	Receptor Tyrosine kinases

1.1 Introduction

Colorectal cancer (CRC) is the third commonest cancer worldwide, with approximately 20% of newly-diagnosed patients already presenting with metastatic disease and 50% of patients developing metastasis in subsequent months or years. The median overall survival (OS) is around 20 months [1–5].

The outlook of patients with metastatic colorectal cancer (mCRC) has been advanced by the introduction in the clinical practice of cetuximab and panitumumab, two monoclonal antibodies (moAbs) that inhibit the epidermal growth factor receptor (EGFR/ErbB1/HER1). These agents are typically administered in combination with chemotherapy in the second- or third-line treatment of individuals who have become resistant to previous rounds of cytotoxic chemotherapy [6–8]; in this chemorefractory setting, patients achieve an objective response and disease stabilization rates of approximately 10% and 20%, respectively [7–9]. Different from other tumor types, such as non-small cell lung cancers (NSCLCs) or melanomas, in which actionable targets such as EGFR or BRAF are constitutively hyperactive as a consequence of underlying genetic alterations [10, 11], mutational abnormalities in the *EGFR* gene are extremely infrequent in colorectal tumors (see below).

The 70% of CRC tumors that are intrinsically refractory to EGFR blockade display primary (also known as innate) resistance. Acquired (or secondary) resistance refers to disease progression in the face of an ongoing treatment that was initially effective. In both primary and secondary resistance, lack of response can be

explained by compensatory signaling activities driven by mutational events or adaptive mechanisms such as biochemical feedbacks or gene expression changes [12, 13]. In the case of colorectal cancer, acquired resistance typically occurs within 3–18 months after treatment initiation [7, 8]. Starting with seminal observations in 2006–2007 [14, 15], several biomarkers of primary resistance to anti-EGFR moAbs in mCRC patients have been progressively identified and biologically validated, and some of them are now routinely used to exclude a number of molecularly defined nonresponders from unnecessary treatment [16, 17]. The topic of acquired resistance has received preclinical and clinical focus more recently, with the emergence of new critical information only in the last 5 years.

Here, we will survey the current state of the art on primary and acquired resistance to anti-EGFR moAbs in mCRC, from early mechanistic investigations to clinical applications, and will discuss fresh knowledge on how to improve the response and delay the relapse in mCRC patients. This chapter is inspired, with relevant updates, to a review article that we have recently authored [18].

1.2 The Genomic Landscape of Resistance to Anti-EGFR Antibodies in Patients with Metastatic Colorectal Cancer

EGFR is a member of the ErbB family of receptor tyrosine kinases (RTKs), which also includes HER2/neu (ERBB2), HER3 (ErbB3) and HER4 (ErbB4) [19]. Following homo- and hetero-dimerization of EGFR with itself or other ErbB members, induced by EGF or other EGF-like ligands, several downstream signal transduction pathways are activated, including the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR axes, but also SRC-like family kinases, PLCγ-PKC, and STATs [19, 20]. Such activation stimulates key processes involved in tumor growth and progression, including proliferation, survival, angiogenesis, invasion, and metastasis [21] (Fig. 1.1).

Of note, the *EGFR* gene is very rarely mutated or amplified in CRC. Because 'addiction' to the EGFR pathway does not have genetic underpinnings, this dependency may represent an aberrant declination of para-physiological traits typical of normal colonic tissues. In the adult intestine, mucosal renewal after tissue damage is prompted by increased EGFR signaling (through transcriptional induction of the receptor and autocrine production of the cognate ligands) [22–24], and is impaired by EGFR inhibition [22]. Importantly, EGFR neutralization curbs the propensity of epithelial cells to undergo neoplastic transformation promoted by inflammatory stimuli [25]. Altogether, these observations suggest that persistent upregulation of EGFR activity during chronic intestinal inflammation—a condition that typically predisposes to colorectal cancer—may act as a pro-tumorigenic cue. This stimulation would positively select for cancer cells relying on EGFR-driven signals for their growth, explaining why a fraction of CRCs are strictly dependent on EGFR activity even in the absence of underlying genetic alterations. On this ground, it

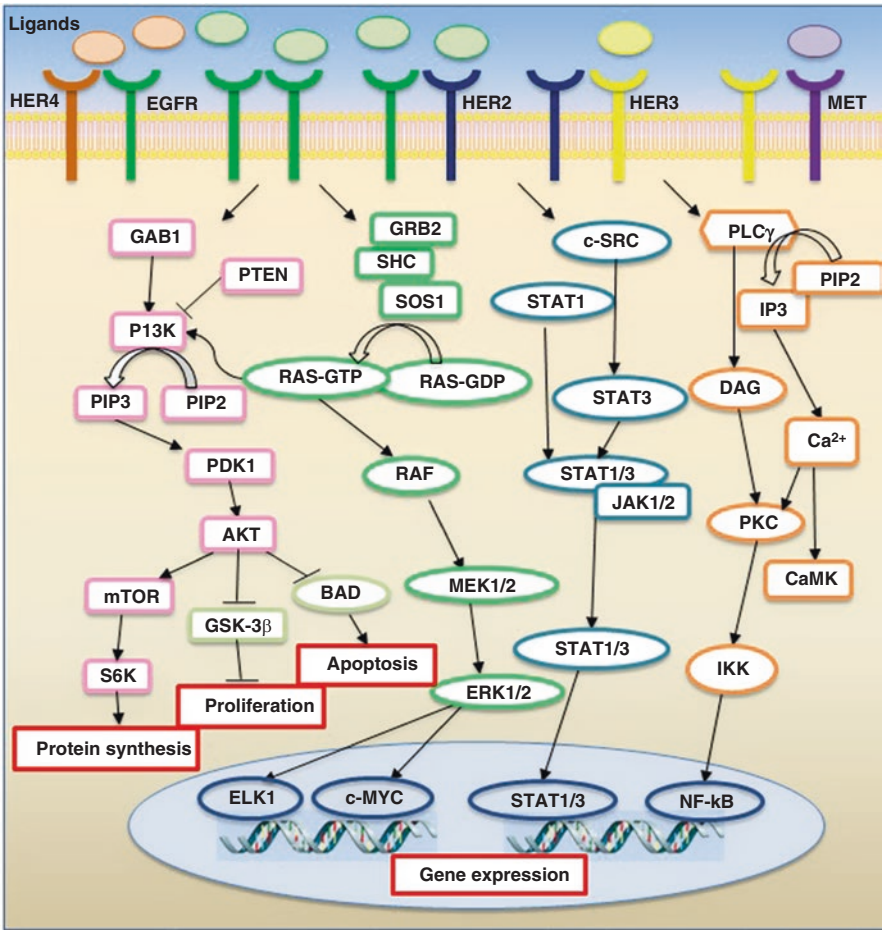

Fig. 1.1 EGFR signaling pathways. (**a**) Following ligand binding and the ensuing receptor homo- and hetero-dimerization, ErbB family members trigger several signaling pathways, including the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR axes, the SRC family kinases, PLCγ-PKC, and STATs. All these signals stimulate cell proliferation and/or survival

comes with no surprise that the increased expression of EGFR and EGFR ligands not only encourages intestinal regeneration during inflammation, but also characterizes 'EGFR-addicted' tumors with marked sensitivity to EGFR inhibition [26–28].

In the absence of genetic alterations correlating with sensitivity to anti-EGFR antibodies, patient stratification is only applied by subtraction: in general terms, the commonest mechanisms of innate resistance involve genomic alterations affecting EGFR downstream effectors, such as *KRAS/NRAS* and *PIK3CA* mutations, with consequent constitutive pathway hyperactivation. The RAS and PI3K signaling

cascades can also be triggered by upstream RTKs other than EGFR [29], leading to an oncogenic shift [30]. In this situation, the primary drug target remains unaltered and continues to be inhibited while an alternative signal transducer becomes activated, circumventing the effects of EGFR inhibition [13, 31] (Fig. 1.2a–c).

It is becoming increasingly clear that tumors can contain a high degree of mutational heterogeneity within the same lesion [32]. Thus, secondary resistance can arise not only through stochastic acquisition of *de novo* genetic lesions along treatment, but also through therapy-induced selection of intrinsically resistant minor subclones already present in the original tumor [33]. If secondary resistance can be re-interpreted as the emergence, under drug pressure, of rare tumor subpopulations featuring primary resistance, then the molecular mechanisms of primary and acquired resistance are expected to be the same. Accordingly, hereinafter we will delineate resistance predictors as absolute traits, specifying, for each determinant, how it contributes to primary or secondary resistance. We will also concentrate on current research efforts that have put forward alternative strategies to bypass such resistances in patients with no other therapeutic options. Table 1.1 summarizes the main predictors of primary and acquired resistance observed in mCRC patients and describes potential approaches for tackling them therapeutically.

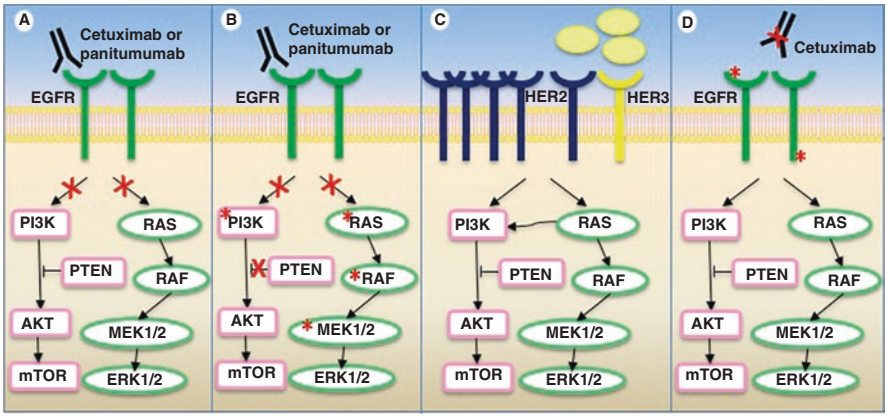

Fig. 1.2 Mechanisms of resistance to anti-EGFR moAbs in mCRC. (**a**) By binding the extracellular domain of EGFR, both cetuximab and panitumumab prevent ligand-induced activation of downstream signaling. (**b**) Activating mutations of genes encoding EGFR transducers such as *KRAS* (by either point mutations or gene amplification), *BRAF*, *PIK3CA* and *MAP2K1* (MEK1), or *PTEN* loss of function, cause relentless activation of downstream signaling that circumvent EGFR inhibition. (**c**) Excessive activation (by either receptor gene amplification or high ligand expression) of alternative receptors, such as HER2 or MET (not shown), can substitute for EGFR inhibition and activate downstream pathways. (**d**) Additional genetic alterations within the target receptor may abolish antibody binding (EGFR extra-cellular domain mutations) or mediate EGFR activation even in the presence of the drug (kinase domain mutations)

Table 1.1 Mechanisms of primary and acquired resistance to anti-EGFR moAbs in mCRC patients and alternative therapeutic approaches

Biomarker	Scientific approach	Alternative strategies proposed	References
Primary resistance			
KRAS mutations	*KRAS* mutant cell lines *in vitro* and *in vivo*	Combination of EGFR and MEK inhibitors was more effective than either agent alone in reducing cell viability *in vitro*	[15]
		Combination of dasatinib (Src kinase inhibitor) with cetuximab induced tumor growth delay but not regression *in vivo*	[60]
	Synthetic lethal interactions in *KRAS* mutant cell lines	Combined IGF-IR and MEK inhibition induced partial tumor regression *in vivo*	[58]
		TAK1 inhibition promoted apoptosis in KRAS-dependent APC-mutant CRC cells and tumor regression *in vivo*	[57]
		Proteasome and topoisomerase inhibitors selectively impaired cell viability (GATA2 and CDC6 could be potential new targets)	[53]
		Combined BCL-XL and MEK inhibition promoted tumor regression *in vivo*	[56]
	Patient-derived xenografts of *RAS* mutant CRCs	Inhibition of MEK and PI3K/mTOR induced tumor growth delay but not regression. This strategy may retard progression in patients	[52]
BRAF mutations	*KRAS* or *BRAF* mutant cells, mouse xenografts and GEMMs.	Combined targeting of BCL-2/BCL-XL and TORC1/2 induced selective apoptosis *in vitro* and tumor regression *in vivo*	[59]
	BRAF V600E CRC models	Combined BRAF and EGFR inhibition was synergistic *in vitro* and *in vivo*	[62, 68, 69]
		Calfizomib (proteasome inhibitor) reduced cell viability *in vitro* and suppressed tumor growth *in vivo*	[74]
	Cell lines with concurrent *PIK3CA* mutations or PTEN loss/BRAF V600E GEMMs	Combination therapy with BRAF and PI3K inhibitors induced apoptosis in vitro, delayed tumor growth *in vivo* and caused tumor regression in GEMMs	[70, 72, 73]
	"BRAF-like" CRC cell lines	"BRAF-like" CRC cell lines were selectively sensitive to the microtubule poison vinorelbine both *in vitro* and *in vivo*	[76]
PIK3CA mutations or PTEN loss	Cells carrying *PIK3CA* mutations or *PTEN* loss but not *BRAF/KRAS* mutations	Everolimus (mTOR inhibitor) slowed cell growth *in vitro* and resulted in long term-tumor growth arrest *in vivo*	[92]
	Analysis of NHS and HPFS studies and VICTOR trial	Adjuvant low-dose aspirin in *PIK3CA*-mutant patients improved survival. Further prospective studies are required	[98, 99]

HER2 amplification	*HER2*-amplified patient-derived xenografts	Combination of cetuximab/pertuzumab with lapatinib induced overt long-lasting tumor regression	[105]
	HERACLES clinical trial	Combination of trastuzumab and lapatinib was active in heavily pretreated mCRC patients	[110]
HER2 mutations	CRC cell lines and patient-derived xenografts with *HER2* mutations	Dual HER2-targeted therapy with trastuzumab and small-molecule inhibitors such as lapatinib or neratinib produced durable tumor regression	[108]
MET activation	HGF-overexpressing cells	Co-treatment with cetuximab and MET inhibitors induced marked tumor regression	[124]
	MET amplified patient-derived xenografts	MET inhibition achieved long-lasting abolition of tumor growth *in vivo*	[119]
EGFR mutation	Patient-derived xenografts with the *EGFR* kinase domain mutation V843I	Combination of cetuximab and afatinib induced marked and long-lasting inhibition of tumour growth	[128]
FGFR1 amplification	Patient-derived xenografts with *FGFR1* amplification	Combination of cetuximab with the selective FGFR kinase inhibitor BGJ398 durably suppressed tumor growth	[128]
PDGFRA mutation	Patient-derived xenografts with the *PDGFRA* R981H mutation	Combination of cetuximab with the PDGFR inhibitor imatinib exerted strong, but short lived, anti-tumor activity	[128]
MAP2K1 (MEK1) mutation	Patient-derived xenografts with the *MAP2K1* K57N mutation	Vertical blockade of MEK and ERK resulted in strong inhibition of tumor growth	[128]
Acquired resistance			
RAS/BRAF activation	CRC cell lines with acquired *KRAS/BRAF* point mutations and/or KRAS amplification and one patient-derived xenograft	Combinations of cetuximab with pimasertib (MEK inhibitor) induced moderate tumor shrinkage *in vivo*	[48]
	Cetuximab sensitive CRC cell lines and patient-derived xenografts	Dual blockade of EGFR and MEK delays the onset of acquired resistance driven by the RAS/MAPK pathway	[61]
HER2 activation	Cells with high heregulin levels or *HER2* amplification	Pertuzumab/lapatinib restored sensitivity to cetuximab *in vitro*	[106]
MET activation	*MET* amplified patient-derived xenografts	Combined inhibition of MET and EGFR induced long-lasting disease stabilization *in vivo*.	[119]
EGFR mutations	Mutations in the *EGFR* ectodomain (S492R, G465E and G465R) found in patients.	Panitumumab remained active in a patient with S492R mutation, which abrogated cetuximab binding. Anti-EGFR monoclonal antibody mixtures or oligoclonal antibodies displayed strong antitumour activity in patient-derived cell cultures and xenografts with *EGFR* mutations in the G465 residue	[126, 128, 129]

1.2.1 RAS

The RAS family includes three small G proteins (KRAS, NRAS, and HRAS) that couple EGFR to downstream activation of the RAF-MEK-ERK pathway [30]. Several retrospective trials have linked *KRAS* mutations in exon 2 (codons 12 and 13), which are found in approximately 40–45% of CRCs [17, 34], to primary resistance to cetuximab or panitumumab [14, 35–37]. The robust predictive significance of such correlations was sufficient for the regulatory approval of companion diagnostics for routine assessment of *KRAS* exon 2 mutations, and now the clinical use of anti-EGFR moAbs is limited to the subset of patients with *KRAS*-wild-type colorectal cancers [34, 38–42].

Although the exclusion of patients with *KRAS* (exon 2)-mutant tumors from anti-EGFR therapy has increased the percentage of responders from 10% to 13–17%, most *KRAS* (exon 2) wild-type tumors remain insensitive to anti-EGFR moAbs [34, 40]. Rare mutations of *KRAS* in codons other than 12 and 13, as well as mutations of *NRAS*, have been found to correlate with therapeutic refractoriness. The relatively high cumulative frequency of such additional mutations, and their successful validation as resistance biomarkers in prospective trials, strongly call for systematic evaluation of these genotypes in clinical practice to enlarge the fraction of patients to be spared anti-EGFR therapy [43]. A very low frequency of *KRAS* amplification (0.7%) has also been reported and demonstrated to correlate with primary resistance [44].

RAS activating mutations and gene copy number gains are responsible not only for primary resistance but also for acquired resistance in 40–60% of patients who progress on cetuximab or panitumumab [45–47]. As mentioned above, such mutations are either pre-existing in minor tumor subclones before treatment initiation [45, 46] or arise as *de novo* alterations under drug pressure [46, 47]. *KRAS* mutations could be detected non-invasively 5–10 months before radiographic evidence of disease progression by analyzing cell-free circulating tumor DNA (ctDNA) [45, 46]. Using this methodology, two recent studies have documented the emergence of several independent clones displaying heterogeneous patterns of *KRAS* and *NRAS* mutations in concomitance with progressive desensitization to EGFR blockade [48, 49].

At present, patients with *KRAS*-mutant mCRC are treated with chemotherapy (with or without anti-angiogenic therapy) and, in the chemorefractory setting, with the multi-target inhibitor regorafenib [50, 51]. To date, direct pharmacologic blockade of the mutant KRAS protein has been unsuccessful; therefore, preclinical studies have concentrated on approaches as different as targeting downstream effectors such as MEK and PI3K [52], leveraging synthetic lethal interactions [53–58], or deploying high-throughput drug screens [59]. Most of these attempts showed that the combinatorial inhibition of two different pathways induces some anti-cancer effects in *KRAS* mutant CRC mouse models, albeit seldom with manifest tumor shrinkages [60] (see Table 1.1). Some of these preclinical strategies have been translated in recently completed phase I/II clinical trials (NCT01085331; NCT01390818;

NCT02039336), for which the results are eagerly awaited. In the case of acquired resistance due to *RAS* mutations, preclinical evidence suggests that combination therapies *ab initio* with EGFR and MEK inhibitors could delay or reverse the emergence of resistance [48, 61].

1.2.2 BRAF

Point mutations of *BRAF*, which encodes a serine/threonine kinase directly activated by RAS and impinging on the downstream effector MEK, are found in 4–13% of advanced CRCs and are typically mutually exclusive with *KRAS* mutations [17, 62].

The *BRAF* V600E mutation has been described as a determinant of poor response to cetuximab and panitumumab [15, 17, 62, 63]. However, the negative predictive power of *BRAF* mutations is undermined by their low frequency and is further biased by the pervasive role of mutant *BRAF* as a negative prognostic biomarker [41, 62–64]. Overall, the predictive impact of this alteration remains to be established and requires further prospective evaluation before clinical applicability [17, 41, 62, 65].

Unlike RAS, BRAF can be efficiently blocked by clinically approved compounds; BRAF small-molecule inhibitors are extensively and successfully used in *BRAF*-mutant melanoma, for example, with response rates (RRs) ranging between 48% and 67% [10, 66]. However, selective BRAF inhibitors such as vemurafenib have failed in *BRAF*-mutant CRCs (RR of 5%) [67]; this lack of efficacy has been ascribed to rapid feedback activation of EGFR following BRAF inactivation, resulting in constitutive signaling through the MAPK–ERK pathway and continued tumor cell proliferation [68, 69]. Accordingly, preclinical studies have demonstrated that BRAF blockade can resensitize to anti-EGFR antibodies [62, 68–70]. At the clinical level, interim reports from an ongoing clinical trial have shown 22% RRs in patients with *BRAF*-mutant mCRC treated with a combination of cetuximab and encorafenib, an investigational BRAF inhibitor [71]. The trial has now entered a phase II expansion cohort (NCT01719380). Investigators are also collecting tumor and blood samples from patients before and after treatment to analyze the drugs' pharmacodynamic consequences, while a broad genomic survey is planned to identify predictive biomarkers [71]. Other combinatorial approaches under preclinical or clinical evaluation [59, 72–74] are listed in Table 1.1.

Intriguingly, some *BRAF* wild-type CRCs display a gene expression signature and a clinical behavior (poor prognosis) that are very similar to those typifying *BRAF*-mutant tumors [75]. By applying a loss-of-function genetic screen, cell lines from this specific tumor subtype were shown to have defects in microtubule formation, unveiling a potential vulnerability to microtubule-disrupting agents [76].

BRAF mutations could be also captured non-invasively by ctDNA analysis, together with concomitant *KRAS* and *NRAS* mutations [48, 49], in patients who had responded to anti-EGFR antibodies and then progressed. Hence, the emergence of *BRAF* mutant subclones may also sustain acquired resistance.

1.2.3 PI3K-AKT-PTEN Pathway

PI3Ks include different classes of lipid kinases; in particular, activation of class IA PI3Ks can be triggered by upstream stimulation from RTKs [77], but also through RAS intermediation [78] or signaling from G protein-coupled receptors [19].

Class IA PI3Ks are heterodimeric proteins composed of a regulatory (p85) and a catalytic (p110) subunit [79]. Activating mutations of *PIK3CA* (encoding p110α) have been detected in 10–20% of CRCs [17, 80–82]; most of them occur in exons 9 and 20, respectively, in the helical and kinase domain [80, 83]. In a retrospective analysis of 110 mCRC patients treated with cetuximab or panitumumab, a statistically significant association between primary resistance to EGFR inhibition and *PIK3CA* mutations (11 in exon 20 and 4 in exon 9, all in *KRAS* wild-type tumors) was reported [84]. Another study, conducted in a patient cohort with a higher prevalence of exon 9 mutations, did not confirm such a correlation [82]. These discrepant data were then reconciled by a retrospective consortium analysis on a larger collection of 1022 tumor samples; the consensus is now that, in the *KRAS* wild-type subpopulation, only the *PIK3CA* exon 20 mutations may be predictive of lack of response to anti-EGFR moAbs [17]. This study also highlighted a strong association between *PIK3CA* exon 9 (but not exon 20) mutations and *KRAS* mutations, reinforcing the notion that *PIK3CA* exon 9 mutations do not have an independent predictive value for anti-EGFR antibody efficacy.

Loss of function of PTEN, a phosphatase that contrasts PI3K activity, occurs in 30% of CRCs through various mechanisms including gene deletion, frameshift or nonsense mutations, and promoter methylation [85, 86]. PTEN inactivation (usually evaluated as lack of protein expression) has been associated with poor sensitivity to anti-EGFR moAbs in mCRC patients in several studies [16, 85, 87, 88], whereas others have only put forward a prognostic role [63]. All in all, both *PIK3CA* exon 20 mutations and PTEN inactivation are promising predictors of reduced responsiveness to anti-EGFR therapies. However, due to the low incidence of exon 20 mutations (2–5%) [89] and lack of an established method for assessment of PTEN inactivation [17, 85, 88, 90, 91], further prospective trials and methodological efforts are necessary to validate the clinical utility of PI3K pathway activation as a negative response determinant.

In principle, patients with tumors exhibiting *PIK3CA* mutations or PTEN loss of function, without concomitant *KRAS/BRAF* mutations, may respond to therapies targeting PI3K or PI3K-downstream transducers, such as mTOR or AKT [92]; however, clinical data have demonstrated only minimal single-agent activity of such therapies at tolerated doses [93–95]. Since the PI3K/AKT inhibition is commonly counteracted by feedback activation of tyrosine kinase receptors [96], it is expected that blockade of the PI3K pathway will provide greater benefit when combined with RTK inhibitors [97]. Phase I/II studies testing mTOR inhibitors, such as everolimus or temsirolimus, in combination with RTK inhibitors or anti-EGF moAbs (in some cases, in the presence of a chemotherapy backbone) are presently being conducted or have been recently completed in mCRC patients (NCT01154335; NCT01139138; NCT01387880; NCT00827684).

Finally, prevention studies have shown improved survival by low-dose aspirin in patients with *PIK3CA*-mutant CRC [98–100]; this observation, which demands further prospective evaluation, could be at least partially related to the fact that the PI3K-AKT axis induces NF-κB-dependent transcriptional upregulation of COX2, which has been demonstrated to exert pro-survival signals in CRC cells [100–102]. Therefore, a *PIK3CA*-mutant makeup may render CRC cells vulnerable to apoptosis by aspirin-mediated COX2 inhibition.

Recently, the presence of *PIK3CA* mutations has been also detected in tissue samples from mCRC patients treated with cetuximab who relapsed while on treatment. Of note, such mutations coexisted with other acquired mutations (in *KRAS, NRAS* or *BRAF* genes) within the same sample [103].

1.2.4 HER2

When considering the cumulative frequency of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* alterations, approximately 60–65% of anti-EGFR resistant cases can be ascribed to the presence of such mutations [16]; in the remaining 30% of 'quadruple negative' cases, still-unidentified features sustain lack of response.

HER2 is the only member of the ErbB family that is not bound by growth factor ligands; it is activated through hetero-dimerization with other ligand-stimulated receptors [20], with the most powerful growth-promoting cues generated by HER2-HER3 heterodimers; *HER2* overexpression, usually caused by gene amplification, enables HER2 constitutive signaling regardless of the activation state of the other partners [104].

Several preclinical and clinical studies have shown that *HER2* amplification is a predictor of poor sensitivity to anti-EGFR antibodies [105, 106]. Based on genotype-response correlations in a platform of patient-derived mCRC tumorgrafts, *HER2* amplification was found to be significantly associated with resistance to cetuximab and specifically enriched in the quadruple negative population [91]. Aberrant HER2 signaling (by either *HER2* amplification or overproduction of the HER3 ligand heregulin) was confirmed as a mediator of lack of response in an independent report [106]. In retrospective clinical studies, patients with colorectal tumors displaying *HER2* amplification or heregulin overexpression and treated with cetuximab or panitumumab had shorter progression-free and overall survival compared with patients with *HER2* wild-type tumors [105–107]. Notably, in patients with acquired resistance, *HER2* amplification was detected in a small fraction (14%) of pretreatment tumor cells and in a much larger proportion of cells (71%) in samples biopsied after anti-EGFR therapy. Similarly, heregulin levels, as assessed in both plasma and tumor specimens, were found to be significantly higher in patients who had relapsed on anti-EGFR therapy with respect to responders [106]. Hence, increased HER2 signaling drives both primary and acquired resistance.

Besides *HER2* amplification, also *HER2* activating point mutations can confer resistance to EGFR blockade in CRC cell lines and patient-derived tumorgrafts [108]. In both instances (amplification and mutations), monotherapy with either anti-HER2

antibodies or HER2 small-molecule inhibitors was not sufficient to induce regression of patient-derived tumorgrafts in mice, and only a combination of antibodies and chemical inhibitors led to massive tumor shrinkage [108, 109]. At least for *HER2* amplification in CRC, trastuzumab (the prototypical anti-HER2 antibody) alone was found to be mainly active against HER3, with minor inhibitory effects on HER2 and EGFR. In contrast, the reversible HER2 small-molecule inhibitor lapatinib prompted rapid and drastic dephosphorylation of all ErbB receptors, but also led to delayed reactivation of HER3 as a compensatory mechanism. Indeed, the stronger effect of the antibody-small molecule combination was attributed to the ability of trastuzumab, through preferential targeting of HER3, to prevent lapatinib-induced HER3 rephosphorylation [109].

These preclinical findings encouraged the design and execution of HERACLES, a clinical trial that assessed the efficacy of the trastuzumab-lapatinib combination in mCRC patients with *KRAS* wild-type, *HER2*-amplified, cetuximab-resistant tumors. Eight (30%) patients achieved objective responses, and 12 (44%) had stable disease [110]. Because this patient subpopulation was heavily pretreated and resistant to both conventional chemotherapy and anti-EGFR antibodies, the outcome data are particularly compelling and testify to the potential of HER2 as a viable target in the treatment of colorectal cancer.

Active HER2 also exacerbates the oncogenic properties of *HER3* mutations, which have been recently described in about 11% of colon cancers [111]. One could envision a ‘dosage effect’ whereby low-grade *HER2* amplification or low levels of heregulin, which alone would not be enough to foster therapeutic resistance, might in fact attenuate sensitivity to EGFR inhibition by cooperating with co-existing *HER3* mutations. Investigational anti-HER3 antibodies and small molecules have been shown to productively contrast HER3-mediated signals and tumor progression in preclinical studies *in vivo* [111] and are now being tested clinically. Therefore, *HER3* mutations in CRC merit investigation as new potential biomarkers of resistance to anti-EGFR treatment as well as new predictors of response to other therapeutic options.

1.2.5 MET

Similar to EGFR family members, the MET tyrosine kinase receptor for hepatocyte growth factor (HGF) can activate growth, survival and motility pathways through the RAS- ERK cascade, the PI3K-AKT axis, and stimulation of SRC and STAT [112–114]. Excessive MET signaling may occur by several mechanisms, including genetic abnormalities such as *MET* amplification and exon 14 skipping mutations (splicing variants that result in the deletion of a negative regulatory domain of the MET kinase), but also as a consequence of increased HGF expression/activity [96]. When genetically altered, *MET* can act both as a primary oncogenic driver and as a determinant of resistance to EGFR tyrosine kinase inhibitors, in particular in NSCLCs harboring *EGFR* mutations [115–117]. *MET* amplification also sustains tumorigenesis and correlates with response to MET small-molecule inhibitors in gastroesophageal cancer [118].

In CRC, *MET* amplification has been documented as a mechanism of primary and acquired resistance to cetuximab and panitumumab [119]. In retrospective analyses, *MET* amplification was detected in around 1% of mCRC samples, in line with previous findings [120]. However, this frequency increased to 12.5% in a subgroup of cetuximab-resistant patient-derived tumorgrafts with wild-type forms of *KRAS, NRAS, BRAF, PIK3CA* and *HER2*. Notably, MET-mediated resistance appears to be driven by a dosage effect: only focal, high-grade amplification of the *MET* locus correlated with overt therapeutic refractoriness, whilst tumors with modest gene copy number gains or polysomy of chromosome 7, where the *MET* gene is located, were still susceptible to cetuximab [120]. Preclinical trials in MET-positive xenografts from CRC cell lines and patient-derived materials revealed that MET inhibition, with or without concurrent interception of EGFR, led to long-lasting abolition of tumor growth [119, 121]. In this vein, a phase II clinical trial aimed to assess the efficacy and safety of the dual MET-ALK inhibitor crizotinib in patients with solid tumors (including CRCs) harboring *MET* genetic alterations has been designed and is currently recruiting participants (NCT02034981).

MET amplification was also found in the tumors of three out of seven patients who had developed a form of acquired resistance to the anti-EGFR antibodies that could not be ascribed to the emergence of secondary *KRAS* mutations. Importantly, the *MET* amplicon was detected in circulating, cell-free DNA as early as 3 months after treatment initiation, well before relapse was observed radiologically. Similar to *HER2* amplification and *KRAS* mutations, rare *MET*-amplified cells could be identified in pre-treatment tumor material from one out of three patients with MET-dependent acquired resistance, suggesting that pre-existing subclones were positively selected under the pressure of anti-EGFR therapy [119].

A recent case report suggests that *MET* amplification in CRC not only precludes sensitivity to upstream EGFR blockade, but also prevents responsiveness to agents targeting the downstream RAS pathway. A patient with a *BRAF*-mutant mCRC who had initially responded to combined EGFR and BRAF inhibition progressively developed resistance. Genetic analysis of matched biopsies before and after therapy revealed a higher representation of *MET*-amplified cancer cells in the post-treatment tissue, and dual blockade of both BRAF and MET proved to be clinically effective [122]. Again, these results point to MET hyperactive signaling as a pervasive resistance trait in mCRC, and highlight the value of MET therapeutic targeting to oppose disease progression.

MET activation can attenuate sensitivity to cetuximab also as a consequence of paracrine HGF stimulation, as observed in CRC cell lines [119, 123] or, more recently, in CRC spheroids enriched in cancer stem cells [124]. In these studies, only concomitant inhibition of both MET and EGFR substantially regressed tumors *in vivo*. This experimental evidence might have clinical relevance, as HGF overexpression correlates with reduced sensitivity to cetuximab in patients [124]. However, the definition of cut-offs to dichotomize HGF-positive versus HGF-negative tumors in the clinic is not trivial, which undermines the portability of assessing HGF levels for patient stratification.

1.2.6 EGFR

Additional genetic alterations within the target oncoprotein, which affect drug binding thus preventing kinase inhibition, are frequently responsible for both primary and acquired resistance in cancer; an emblematic example is represented by the T790M 'gatekeeper' secondary mutation in the *EGFR* gene, which drives resistance to first-generation EGFR small-molecule inhibitors in *EGFR*-mutant NSCLC [125]. In colorectal cancer, different mutations in the extracellular domain of EGFR have been recently described as a typical mechanism of acquired resistance, namely, S492R, G465E and G465R mutations [126–128] (Fig. 1.2d). Structural analyses indicate that while S492 selectively lies in the cetuximab binding site, G465 is located in the center of the region in which the epitopes of both cetuximab and panitumumab overlap. Accordingly, S492R abrogates cetuximab binding but retains panitumumab interaction, whereas G465E and G465R prevent binding of both antibodies. Studies in patient-derived tumorgrafts [128] and cell cultures [129] harboring mutations in the G465 residue have shown that new-generation anti-EGFR antibodies that bind EGFR epitopes different from those recognized by cetuximab and panitumumab are very effective in opposing the growth of these tumors.

Resistance may be also driven by mutations in the EGFR kinase domain: two alterations have been identified as circulating mutations by cell-free DNA analysis [49], and one has been detected in cetuximab-resistant patient-derived tumorgrafts [128]. Treatment of such tumorgrafts with an EGFR small-molecule inhibitor or cetuximab alone was not effective, but the combination resulted in substantial and durable inhibition of tumor growth [128].

1.3 Newly Emerging Biomarkers of Drug Resistance and Sensitivity

A recent systematic survey of molecularly annotated patient-derived tumorgrafts has functionally linked therapeutic responses to EGFR inhibitors with complete exome sequence and copy number analyses as a way to identify new resistance traits and, potentially, new druggable targets. By doing so, in addition to the genetic abnormalities described above, new alterations have been found, including mutations/amplification in *FGFR1*, *PDGFRA* and *MAP2K1* [128] and outlier overexpression of *IGF2* [28]. All these tumorgrafts proved to be susceptible to therapies targeting the resistance-conferring genetic alterations. Another actionable lesion in CRC that has recently received clinical attention is the *NTRK1* chromosomal rearrangement, which leads to the synthesis of a highly expressed fusion protein with constitutive NTRK kinase activity. A case report has described a patient with metastatic colorectal cancer harboring an *LMNA–NTRK1* rearrangement who achieved a remarkable clinical and radiographic response to entrectinib (RXDX-101), a multikinase inhibitor targeting TRK, ALK, and ROS1, which was followed by the

emergence of resistance [130]. Longitudinal monitoring of the *LMNA–NTRK1* status by ctDNA analysis revealed the acquisition of two novel NTRK1 kinase domain mutations (G595R and G667C) that were absent from ctDNA collected at the time of treatment initiation. According to structural studies, such mutations are expected to abrogate or reduce entrectinib binding to the catalytic pocket, rendering tumors less vulnerable to this specific inhibitor [131].

While the quest for resistance biomarkers has yielded considerable results in the past years, data remain immature as far as the identification of positive determinants of responsiveness to EGFR blockade is concerned. As noted above, *EGFR* is very rarely mutated or amplified in CRC, and the only known means to achieve EGFR hyperactivation seems to be increased paracrine/autocrine expression of some EGFR ligands, in particular amphiregulin and epiregulin. Accordingly, high levels of amphiregulin and epiregulin correlate with a better response to anti-EGFR moAbs [26, 27, 29, 132, 133]. However, as already discussed for HGF, the clinical application of this information is hindered by the difficulty in setting thresholds to distinguish ligand-positive versus ligand-negative tumors. Intriguingly, responsive cases appear to be enriched for genetic lesions (mutations or amplification) of *IRS2*, a cytoplasmic adaptor protein that relays signals from tyrosine kinase receptors to downstream effectors [128]. In functional assays, RNA interference-mediated silencing of IRS2 was accompanied by attenuated sensitivity to cetuximab and reduced activation of EGFR-dependent pathways, in line with the role of IRS2 as an amplifier of tyrosine kinase signals. The clinical applicability of this information for optimized selection of responsive patients remains to be determined.

1.4 Outlook

Although many genetic determinants of resistance to anti-EGFR antibodies have been recently documented, and some of them have been validated as alternative pharmacologic targets, there is still space for the identification of additional druggable alterations and the deployment of further therapeutic strategies. Genome-scale analyses of CRC tumor collections are expected to provide a fresh catalog of new mutations, rearrangements, and copy-number alterations with therapeutically actionable potential [134, 135] and will receive further momentum by proteogenomics data [136]. Moreover, promising results are being offered by treatments that disrupt immune evasion strategies. To stimulate immune suppression, tumor cells often engage immune checkpoint molecules, such as CTLA-4 and PD1, which quench cytotoxic T-cell activation. Antibodies against CTLA-4 (e.g., ipilimumab) or PD1 (e.g., nivolumab, pembrolizumab) have been shown to induce durable tumor regressions [137, 138] in mismatch repair–deficient colorectal cancer, likely because the large number of somatic mutations present in these hypermutated tumors increase the presentation of non-self immunogenic neo-antigens and, hence, sensitize to immune checkpoint blockade [139].

Although several resistance mechanisms have been documented so far, mutant *RAS* is the only clinically validated biomarker for selection of mCRC patients eligible to treatment with anti-EGFR antibodies. This attrition between experimental discovery and clinical implementation advocates the introduction of new clinical trial designs that capitalize on reliable preclinical findings. In this regard, a successful story is our experience with mCRC cases harboring *HER2* amplification: from retrospective identification of this alteration in archival patient material, and after establishing a statistically robust correlation between the occurrence of *HER2* amplification and primary resistance to EGFR inhibition, we moved to testing different therapeutic options in HER2-positive patient-derived tumorgrafts and found one treatment that resulted in overt and long-lasting tumor regression [105, 109]. The very same regimen was then applied to patients with *HER2*-amplified tumors with positive results [110]. In this case, reliable tumor models, stringent endpoint criteria for animal studies, and accurate genetic selection were the ingredients that made this translational effort a winning opportunity.

Future clinical trials will be informed by real-time monitoring of tumor evolution along treatment so as to adjust therapies (likely, combination therapies) to the continuing mutability of cancer. While multi-dimensional analysis of serial biopsies is, in principle, the most informative approach, it should also be considered that an individual tumor biopsy may not be representative of overall intratumor heterogeneity, and post-treatment tumor tissue is difficult to obtain. Such limitations can be overcome by less invasive analyses on ctDNA, which can offer a high degree of sensitivity and specificity to detect the surfacing of resistance-conferring mutations over the course of therapy [49, 140]. The mechanism by which ctDNA is released into the bloodstream and whether multiple metastases, or different regions within the same tumor, shed ctDNA homogeneously are still unclear; however, the proof-of-concept that such an approach is viable and its merit in raising an early warning of acquired resistance are now consolidated [46, 49, 141, 142]. Inevitably, to gather a more comprehensive picture of tumor adaptation to targeted treatment and to more effectively tackle the ever-evolving resistant phenotype at the therapeutic level, mutational analysis needs to be integrated by other molecular approaches that detect changes in gene expression, proteins, and protein activities. While this is feasible, at present, only in bioptic material—with all the hurdles and challenges related to repeated biopsies discussed above—hints are emerging whereby non-invasive techniques may prove useful also to measure RNA and protein/phosphoprotein levels in blood, for example by isolating circulating exosomes [143].

If appropriately dosed in quantity and scheduled in time, new investigational therapies could also leverage tumor heterogeneity to their own advantage: creating a “balance” between drug activity and graded responsiveness of different clones to drug pressure might be useful to retard the onset of resistance and, ideally, to turn cancer into a chronic disease. Intriguingly, the prevalence of *KRAS* mutant subclones that become detectable in the blood of mCRC patients on anti-EGFR therapy has been demonstrated to decline after treatment withdrawal, leaving space to *KRAS* wild-type populations that regain drug sensitivity [142]. This could explain why some mCRC patients benefit from multiple challenges with anti-EGFR antibodies.

More than a decade after the introduction of cetuximab in the treatment of metastatic colorectal cancer, much is known about the genetic determinants of primary and acquired resistance to anti-EGFR moAbs in CRC. What is now becoming increasingly clear is that therapeutic resistance is not a fixed, irreversible state, but rather the expression of a resilient phenotype that reacts to drug pressure through manifold sophisticated elusion strategies. The time is ripe to move from a static vision of the disease to a more flexible appraisal of tumor evolution, adaptation and dynamic instability.

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