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Chapter

Predictive Biomarkers for Monoclonal Antibody Therapies Targeting EGFR (Cetuximab, Panitumumab) in the Treatment of Metastatic Colorectal Cancer

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Abstract

The treatment for patients with metastatic colorectal cancer has progressively improved over the past few decades with the development of more effective anti-cancer drugs and multi-disciplinary management approaches that combine sequential lines of non-cross-resistant drugs and increased use of potentially curative surgery for metastases of the liver and lung. In this setting, the introduction of monoclonal antibody therapies that target the epidermal growth factor receptor (EGFR) (cetuximab and panitumumab) has made an important contribution to improved patient outcomes. However, the efficacy of therapies is generally limited to a small proportion of patients and associated with toxicity and high cost. There is an urgent clinical need for robust predictive biomarkers to guide the effective use of therapy options. In this chapter we review clinical and molecular predictive markers of primary therapy response for metastatic colorectal cancer, focusing on anti-EGFR antibody therapies, discussing both currently approved and emerging biomarkers.

Keywords: metastatic colorectal cancer, epidermal growth factor receptor (EGFR), cetuximab, panitumumab, predictive biomarkers

1. Introduction

Metastatic colorectal cancer (mCRC) remains a major contributor to cancer-related morbidity and mortality worldwide. Among patients diagnosed with colorectal cancer, approximately 20–25% present with distant metastases, while another 20–35% develop metastases following curative-intent treatment for early-stage cancer [1]. The median overall survival for mCRC has improved significantly over the past few decades, increasing from 10 to 12 months for 5-fluorouracil plus leucovorin to currently beyond 30 months [2]. Improvements have been driven by advancements in surgery for metastatic disease, the expansion of chemotherapy options and the introduction of targeted therapies such as monoclonal antibodies against the epidermal growth factor receptor (EGFR) or the vascular endothelial growth factor A (VEGFA) [2]. Presently, there are 11 therapeutics approved by the

United States Food and Drug Administration (FDA) for the treatment of mCRC, including 5-fluorouracil, irinotecan, capecitabine, oxaliplatin, bevacizumab, cetuximab, panitumumab, ziv-aflibercept, regorafenib, ramucirumab, and trifluridine-tipiracil. The expansion of treatment options has resulted in an increased clinical need for predictive biomarkers to guide the effective use of therapy. Only a small proportion of patients will respond to any given therapy, and treatments are associated with significant toxicities and often with high financial costs.

Predictive biomarkers for anti-cancer agents are best developed prospectively as companion diagnostics during the drug development process. However, these can also be developed retrospectively through analysis of samples and data from previously conducted randomized clinical trials. Another avenue for marker discovery are longitudinal studies of patients analyzing the emergence of drug resistant tumor clones, although mechanisms of intrinsic (primary) and acquired (secondary) drug resistance may differ. Predictive markers can provide either drug sensitivity (positive prediction of response) or resistance (negative prediction of response) information depending on the biomarker-drug relationship.

There are many challenges in the biomarker development process, such as the choice of analyte (e.g. urine, blood, tissue), cancer sampling procedures (e.g. circulating tumor cells, primary cancer, metastatic lesions), technology for marker evaluation (e.g. DNA, RNA or protein) and determination of clinically relevant cut-offs. In this chapter, we review development efforts for predictive biomarkers for patients with mCRC focusing on anti-EGFR antibody therapies. Our discussion will concentrate on markers of primary drug resistance; markers of acquired drug resistance have been summarized in recent reviews [3, 4].

2. Anti-EGFR therapy

EGFR is a tyrosine kinase transmembrane receptor that belongs to the ErbB protein family. EGFR-mediated signaling has important roles in cell proliferation, survival and differentiation, and dysregulation is a central driver in multiple malignancies including colorectal cancer [4–6]. EGFR interacts with multiple ligands including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin (AREG), epiregulin (EPR), betacellulin (BTC), heparin-binding EGF (HB-EGF), epigen (EPN), and neuregulin 1-4 (NRG1-4). Activation of EGFR following ligand binding triggers a variety of signaling cascades, including the RAS/MAPK, PI3K/AKT, PLCγ/PKC, SRC tyrosine kinase and STAT pathways. In addition, ligand binding can induce EGFR translocation to the nucleus where EGFR behaves as a co-transcriptional activator regulating key genes such as Aurora Kinase A (*AURKA*), Cyclin D1 (*CCND1*), Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) and MYB Proto-Oncogene Like 2 (*MYBL2*).

EGFR is overexpressed in colorectal tumors, with most estimates between 40% and 80% depending on the methods and cut-offs used, highlighting the receptor as a prime drug target in this malignancy [7, 8]. Two monoclonal antibodies targeting EGFR have been clinically approved for the treatment of mCRC including cetuximab (Erbitux®), a chimeric mouse-human IgG_1 antibody, and panitumumab (Vectibix®), a humanized IgG_2 antibody. Both antibodies bind the extracellular domain of EGFR, inhibiting ligand-induced tyrosine kinase activation and leading to EGFR cellular internalization and degradation, thereby preventing the activation of downstream signaling (**Figure 1**). Panitumumab has a higher binding affinity for EGFR than cetuximab [9], and cetuximab is thought to additionally lead to activation of the immune response through antibody-dependent cell-mediated cytotoxicity (ADCC) due to the IgG_1 chimeric antibody structure [10, 11]. With respect to

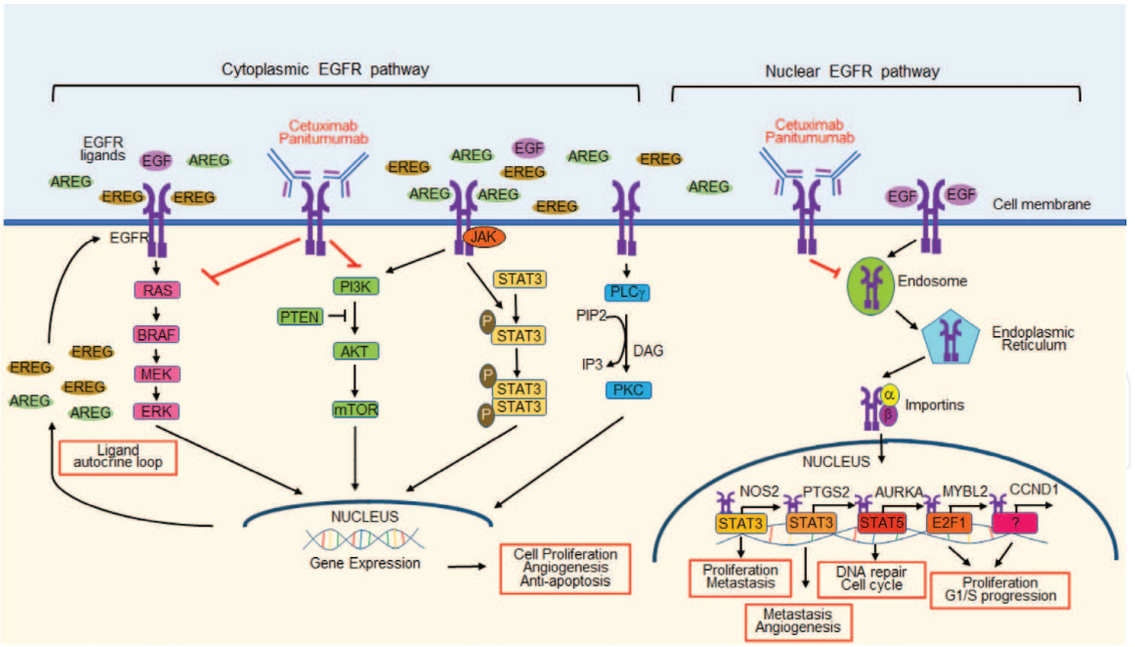

Figure 1.
Targeting of the EGFR signaling pathway with anti-EGFR monoclonal antibodies. EGFR activation is triggered by ligand binding which results in the formation of receptor homo- or hetero-dimers. Receptor autophosphorylation at tyrosine residues within the cytoplasmic tail acts as a docking site for proteins with Src homology2 (SH2) and phosphotyrosine-binding domains (PTB), initiating cellular signaling via the RAS/MAPK, PI3K/AKT, STAT and PLCγ/PKC pathways. Ligand binding can further stimulate EGFR translocation into the nucleus, with nuclear EGFR interacting with transcription factors to drive expression of target genes including NOS2, PTGS2, AURKA, MYBL2 and CCND1. EGFR signaling in tumor cells promotes cell proliferation and survival, and this can be blocked with antibodies against the receptor (cetuximab and panitumumab).

toxicity, panitumumab treatment is associated with significantly lower occurrence of grade 3–4 infusion reactions (allergic reactions) than cetuximab due to its fully humanized nature [12]. Despite these differences, cetuximab and panitumumab showed clinical equivalence in efficacy in refractory patients [12], and both are approved for use in combination with chemotherapy in the first and second line setting or as monotherapy for refractory disease.

In unselected patient populations, the response rate to anti-EGFR therapy is typically less than 30% [13], and for patients who initially respond to treatment most tumors become refractory within 3–12 months [14]. The need to identify biomarkers predictive of EGFR response is therefore vital, and numerous studies have explored resistance mechanisms to EGFR blockade. Findings have unraveled a variety of biomarkers and pathways that are associated with resistance to anti-EGFR therapy. As discussed below, this work has led to the endorsement of predictive testing for tumor *RAS* (*KRAS* and *NRAS*) mutation status and consideration of primary tumor location to guide the use of anti-EGFR therapy. Efforts to discover and validate additional biomarkers is ongoing to further refine treatment delivery are ongoing.

2.1 Current predictive biomarkers for anti-EGFR therapy

2.1.1 KRAS and NRAS mutations

Genes of the RAS type GTPase family, comprising *KRAS*, *NRAS* and *HRAS*, are principal downstream mediators of activated EGFR signaling [15]. In colorectal cancer, *KRAS* and *NRAS* are major oncogenes, with activating mutations found in approximately 40% and 3–5% of cases, respectively [16]. Constitutive downstream signaling through oncogenic RAS proteins activates processes contributing to tumor progression and metastasis, independent of EGFR and other cell surface receptor

kinases [15]. As anticipated from the biological mechanism, mutations in *KRAS* and *NRAS* genes have been found to render tumors insensitive to anti-EGFR therapy.

The majority of *KRAS* mutations (85–90%) in colorectal cancer occur in exon 2 at codons 12 and 13 [16]. Analyses of clinical trials of cetuximab or panitumumab over the last decade have provided conclusive evidence that patients with *KRAS* mutations in exon 2 do not benefit from anti-EGFR therapy when given as a single agent or combined with chemotherapy (**Table 1**) [17–24]. Retrospective analyses of the randomized phase III CO.17 and 20020408 studies which evaluated cetuximab or panitumumab plus best supportive care (BSC) *vs* BSC alone in patients with chemotherapy-refractory mCRC, respectively, found a significant improvement in outcomes for patients with wild-type *KRAS* exon 2 tumors, but no benefit of anti-EGFR therapy in patients who had mutant *KRAS* exon 2 tumors [19, 22]. Similar results for the first-line setting were subsequently reported for both retrospective and prospective analyses of several randomized clinical trials, including the phase II OPUS and phase III PRIME studies which examined cetuximab or panitumumab plus oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) *vs* FOLFOX4 alone, respectively [23, 25], and the phase III CRYSTAL study which assessed the addition of cetuximab to irinotecan, infusional fluorouracil, and leucovorin (FOLFIRI) [18]. Prospective analysis of the randomized phase III 20050181 study which evaluated panitumumab plus FOLFIRI in the second-line setting further confirmed the predictive value of *KRAS* exon 2 mutation status [24].

There is evidence that *KRAS* codon 12 and 13 mutations may exhibit differential biological effects, including variable ratios of these codon mutations between tumor types [16] and weaker *in vitro* transforming activity for *KRAS* codon 13 as compared to codon 12 mutant proteins [26]. Accordingly, some studies have suggested that patients with KRAS glycine (G)-to-aspartate (D) transitions at codon 13 (G13D), the most common codon 13 variant in colorectal cancer, might derive some benefit from anti-EGFR therapy [27, 28]. A retrospective consortium analysis assessing patients with chemotherapy-refractory mCRC treated with cetuximab who participated in multiple clinical trials (CO.17, BOND, MABEL, EMR202600, EVEREST, BABEL and SALVAGE) or who received off-study treatment reported longer overall and progression-free survival among individuals with KRAS G13D-mutated tumors than with other *KRAS*-mutated tumors [27]. An analysis of the updated pooled data sets from the CRYSTAL and OPUS studies also reported that addition of cetuximab to first-line chemotherapy appeared to benefit patients with KRAS G13D-mutant tumors [28]. In contrast, a retrospective analysis of 110 patients treated with cetuximab, found that patients with KRAS G13D mutations were unlikely to respond to therapy [29], and similar findings were reported for a retrospective pooled analysis of three randomized phase III trials evaluating panitumumab therapy (20050203, first line; 20050181, second line; and 20020408, monotherapy) [30]. To resolve this controversy, the randomized phase II ICECREAM study prospectively assessed cetuximab monotherapy and cetuximab plus irinotecan in patients with KRAS G13D-mutated chemotherapy-refractory mCRC. In this study, no statistically significant improvement in disease control was observed for patients with this rare molecular subtype [31].

More recently, several retrospective analyses have indicated that not only *KRAS* exon 2 mutations but also *KRAS* exons 3 and 4 and *NRAS* exons 2, 3, and 4 mutations are negative predictive markers for anti-EGFR therapy [23, 32–36]. These additional mutations are observed in approximately 15–20% of wild-type *KRAS* exon 2 tumors [23, 32]. Reassessment of the randomized OPUS and PRIME studies of cetuximab or panitumumab plus FOLFOX4 *vs* FOLFOX4 alone in the first-line setting found that additional *RAS* mutations predicted a lack of response [23, 34], and corresponding observations were reported for the CRYSTAL study of cetuximab plus FOLFIRI [32]. Accordingly, analyses of single arms of the phase III FIRE-3 study

Study	Treatment arms	Number of patients	PFS (months)	HR PFS	95% CI	p-Value
RAS wild-type						
CRYSTAL	FOLFIRI + C	178	11.4	0.56	0.41–0.76	p < 0.001
	FOLFIRI	189	8.4			
FIRE-3	FOLFIRI + C	199	10.3	0.97	0.88–1.99	0.77
	FOLFIRI +B	201	10.2			
OPUS	FOLFOX + C	38	12	0.53	0.27–1.04	0.0615
	FOLFOX	49	5.8			
PEAK	FOLFOX + P	50	13	0.65	0.44–0.96	0.029
	FOLFOX + B	60	9.5			
PRIME	FOLFOX + P	259	10.1	0.72	0.58–0.90	0.004
	FOLFOX	253	7.9			
20050181	FOLFIRI + P	208	6.4	0.7	0.54–0.90	0.007
	FOLFIRI	213	4.6			
20020408	P + BCS	107	12.3 wks	0.45	0.34–0.59	p < 0.001
	BSC	110	7.3 wks			
CO.17	C + BSC	117	3.7	0.4	0.3–0.54	p < 0.001
	BSC	113	1.9			
RAS mutant						
CRYSTAL	FOLFIRI + C	246	7.4	1.1	0.85–1.42	0.47
	FOLFIRI	214	7.5			
FIRE-3	FOLFIRI + C	n.a	n.a	n.a	n.a	n.a
	FOLFIRI +B	n.a	n.a			
OPUS	FOLFOX + C	92	5.6	1.54	1.04–2.29	0.0309
	FOLFOX	75	7.8			
PEAK	FOLFOX + P	n.a	n.a	n.a	n.a	n.a
	FOLFOX + B	n.a	n.a			
PRIME	FOLFOX + P	272	7.3	1.3	1.07–1.60	p < 0.001
	FOLFOX	276	8.7			
20050181	FOLFIRI + P	299	4.8	0.86	0.71–1.05	0.14
	FOLFIRI	294	4			
20020408	P + BCS	76	7.4 wks	0.99	0.73–1.36	n.r.
	BSC	95	7.3 wks			
CO.17	C + BSC	81	1.8	0.99	0.73–1.35	0.96
	BSC	83	1.8			

Abbreviations: PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; FOL, folinic acid; F, fluorouracil; IRI, irinotecan; OX, oxaliplatin; B, bevacizumab; C, cetuximab; P, panitumumab; n.r., not reported; BSC, best supportive care.

Table 1.
Summary of clinical trials and treatment effects within subgroups defined by RAS status in patients with metastatic colorectal cancer.

evaluating cetuximab plus FOLFIRI and the phase II PEAK study evaluating panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) in the first-line setting reported a more pronounced survival advantage for the wild-type *RAS* population as compared to the wild-type *KRAS* exon 2 population [36, 37]. Retrospective analysis of the randomized 20050181 study of panitumumab plus

FOLFIRI in the second-line setting further found no benefit of panitumumab addition in patients with *RAS* mutations beyond *KRAS* exon 2 [35]. Low response rates for additional *RAS* mutations were also reported by a European consortium analyzing tumor samples from a large cohort of patients with chemotherapy-refractory mCRC treated with cetuximab and chemotherapy [38].

A systematic review and meta-analysis of nine randomized controlled trials for anti-EGFR therapy comprising a total of 5948 participants evaluated for *RAS* mutations has confirmed tumors without any *RAS* mutations to have significantly superior progression-free (PFS) and overall survival (OS) as compared to tumors with *RAS* mutations. No difference in PFS or OS benefit was evident between tumors with *KRAS* exon 2 mutations and tumors with other *RAS* mutations [33]. Treatment guidelines for mCRC now recommend *RAS* testing prior to start of anti-EGFR antibody therapy to exclude patients with mutated *RAS* [2, 21]. However, *RAS* mutations only account for approximately 35–50% of nonresponsive patients, and the search for additional biomarkers that predict resistance continues to be an active area of research as surveyed below.

2.1.2 Primary tumor location

Colorectal cancers can be broadly grouped by their primary tumor location within the colon [39]. The left-sided colon, comprising the distal third of the transverse colon, splenic flexure, descending colon, sigmoid colon and rectum, are derived from the embryonic hindgut. The right-sided colon, comprising the proximal two-thirds of the transverse colon, ascending colon and caecum, is derived from the embryonic midgut. Baseline differences exist along the colorectal tract such as cell composition and function of the epithelium, the microbiome and gene expression. Strong evidence for the prognostic effect of primary tumor location is available from clinical studies in patients with mCRC, with right-sided tumors exhibiting a worse prognosis [40, 41]. Right- and left-sided cancers differ in their clinical and molecular characteristics: right-sided colon cancers are more likely to be diploid and have high-grade or mucinous histology, DNA mismatch-repair deficiency and microsatellite instability, CpG island methylation, *BRAF*, *TGFBR2* and *PIK3CA* mutations [41, 42], while left-sided cancers often show chromosome instability, *APC*, *KRAS*, *SMAD4* and *TP53* mutations [43]. Right-sided tumors have also been associated with more frequent overexpression of the EGFR ligands, EREG and AREG, and amplification of EGFR and human epidermal growth factor receptor 2 (HER2) [44, 45]. In cohort studies, the classification of tumor sidedness is variable, with right-sided tumors commonly defined as comprising the region from the ceacum to the splenic flexure.

Clinically, primary tumor location was not considered of particular interest in metastatic patients treated with anti-EGFR therapy, until the importance of sidedness as a biomarker was recognized. Retrospective surveys of clinical trials have indicated that while anti-EGFR therapy provides clinical benefit to patients with *RAS* wild-type mCRC, this benefit is specific to patients with left-sided tumors (**Table 2**). In the CRYSTAL and FIRE-3 studies of cetuximab in the first-line setting, patients with *RAS* wild-type left-sided tumors had better outcomes compared to the respective comparators (FOLFIRI alone and FOLFIRI plus bevacizumab), while limited efficacy was observed in patients with RAS wild-type right-sided tumors [46]. Benefit from cetuximab treatment specific to patients with *KRAS* wild-type left-sided tumors was further observed for the randomized phase III CALGB/SWOG 80405 study of cetuximab or bevacizumab with either irinotecan/5-FU/leucovorin (FOLFIRI) or oxaliplatin/5-FU/leucovorin (mFOLFOX6) [47]. Similar results for patients with *RAS* wild-type left-sided as compared to right-sided

Study	Treatment arms	Number of patients	PFS (months)	HR PFS	95% CI	p-Value
Left-sided colorectal cancer						
CRYSTAL	FOLFIRI	138	8.9	0.5	0.34–0.72	<0.001
	FOLFIRI + C	142	12			
PRIME	FOLFOX	159	9.2	0.72	0.57–0.90	n.r.
	FOLFOX + P	169	12.9			
CALGB/SWOG 80405	FOLFOX/FOLFIRI + B	152	11.2	0.84	0.66–1.06	0.15
	FOLFOX/FOLFIRI + C	173	12.7			
FIRE-3	FOLFIRI + B	149	10.7	0.9	0.71–1.14	0.38
	FOLFIRI + C	157	10.7			
PEAK	FOLFOX + P	53	14.6	0.65	0.21–2.0	n.r.
	FOLFOX + B	54	11.5			
Right-sided colorectal cancer						
CRYSTAL	FOLFIRI	51	7.1	0.87	0.47–1.62	0.66
	FOLFIRI + C	33	8.1			
PRIME	FOLFOX	49	7	0.8	0.50–1.26	n.r.
	FOLFOX + P	39	7.5			
CALGB/SWOG 80405	FOLFOX/FOLFIRI + B	78	10.2	1.64	1.15–2.36	0.006
	FOLFOX/FOLFIRI + C	71	7.5			
FIRE-3	FOLFIRI + B	50	9	1.44	0.92–2.26	0.11
	FOLFIRI + C	38	7.6			
PEAK	FOLFOX + P	22	8.7	0.84	0.18–3.79	n.r.
	FOLFOX + B	14	12.6			

Abbreviations: PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; FOL, folinic acid; F, fluorouracil; IRI, irinotecan; OX, oxaliplatin; B, bevacizumab; C, cetuximab; P, panitumumab; n.r., not reported; BSC, best supportive care.

Table 2.
Summary of clinical trials and treatment effects within subgroups defined by primary tumor location in patients with metastatic colorectal cancer.

tumors were reported for panitumumab for analyses of the PRIME (comparator: FOLFOX alone) and PEAK studies (comparator: FOLFOX plus bevacizumab) [48]. A meta-analysis integrating these data for the first-line setting is available [49]. For the second-line setting, a retrospective analysis of FIRE-3 study also found evidence of better outcomes for cetuximab treatment in patients with *KRAS* wild-type left-sided tumors as compared to right-sided tumors (comparator: bevacizumab) [50]. Similar results for panitumumab were reported in a preliminary efficacy analysis of the 20050181 study for *RAS/BRAF* wild-type patients (comparator FOLFIRI) [51]. A retrospective analysis of the CO.17 study in the treatment-refractory setting further observed that only individuals with *KRAS* wild-type left-sided tumors appeared to benefit from cetuximab as compared to BSC [52].

Given the above evidence, NCCN guidelines now recommend the use of anti-EGFR antibody therapies for the treatment of *RAS* wild-type left-sided colon cancers only [53].

2.2 Future predictive biomarkers for anti-EGFR therapy

2.2.1 Skin toxicity

Dermatological toxicities such as papulopustular rash (acneiform eruption), erythema, and skin fissures are common side effects of treatment with anti-EGFR antibodies, as EGFR is involved in the normal development and physiology of the epidermis [54]. Both undifferentiated and proliferating keratinocytes in the basal and suprabasal layers of the epidermis express EGFR, and keratinocytes depend on EGFR to regulate proliferation, differentiation, migration, and survival [55]. The emergence of skin toxicity has therefore been investigated as an on-target marker for anti-EGFR therapy efficacy in patients with mCRC.

Subset analyses of outcomes by skin toxicity severity suggest that improvements in outcome are associated with a higher grade of severity for patients treated with either panitumumab or cetuximab. For example, in the CRYSTAL trial of cetuximab as a first-line therapy, PFS was 11.3 months compared with 5.4 months in patients with G3 and G0-1 skin reactions, respectively [56]. Similarly, the randomized phase III EPIC study of cetuximab plus irinotecan *vs* irinotecan after fluoropyrimidine and oxaliplatin failure in patients with EGFR-expressing mCRC observed a median PFS of 15.6 months for patients who developed G3-4 rash compared to 5.8 months for those with no rash [57]. In the PRIME study of panitumumab plus FOLFOX4 (first line) and 20050181 study of panitumumab plus FOLFIRI (second line) the addition of a targeted agent even appeared detrimental for outcomes in patients with G0-1 skin toxicity as compared to the control arms [58, 59]. Better outcomes in patients with higher-grade skin toxicity were further noted for both arms in a randomized trial of cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer [60]. A meta-analysis by Petrelli *et al* of 14 studies including a total of 3833 patients, found that the occurrence of skin toxicity was a predictive factor for survival (HR 0.51; 95% CI 0.40–0.64) and progression (HR 0.58; 95% CI 0.49–0.68). However, 12 of the studies included patients with either *KRAS* wild-type or mutated tumors, and data on skin toxicity by *KRAS* mutation status remains limited.

Analysis of skin toxicity in the randomized phase III ASPECCT study of panitumumab *vs* cetuximab in chemorefractory wild-type *KRAS* exon 2 mCRC observed improved outcomes in patients with higher grade of severity for both antibodies, although patients with higher-grade skin toxicity had longer median duration of treatment [61]. Two retrospective trial analyses (PRIME and AIO CRC-0104 [cetuximab with CAPOX or CAPIRI, first-line]) suggest that the relationship between skin toxicity and outcome may not only apply to patients with wild-type *RAS* tumors, but perhaps also to patients mutant *RAS* tumors [62, 63]. A recent meta-analysis of skin toxicity identified seven and five studies that reported information on PFS and OS stratified by *KRAS* mutation status, respectively [64]. Improved clinical outcome in the presence of higher grade severity was observed for both patients with wild-type *KRAS* tumors and those with mutant *KRAS* tumors (PFS for wild-type *KRAS*, HR = 0.60, 95% CI (0.51, 0.70); mutant *KRAS*, HR = 0.60, 95% CI (0.45, 0.80), OS for wild-type *KRAS*, HR = 0.54, 95% CI (0.46, 0.65), mutant *KRAS*, HR = 0.64, 95% CI (0.50, 0.81), $P < 0.001$]. However, only mCRC patients with wild-type *KRAS* tumors who suffered grade 2+ skin toxicity derived absolute benefit from anti-EGFR treatment additional to best BSC or chemotherapy (PFS HR = 0.58, 95% CI (0.41, 0.82), OS HR = 0.73, 95% CI (0.61, 0.88)).

These data raise the question whether wild-type *RAS* patients receiving anti-EGFR therapy who do not develop skin toxicity should receive a dose escalation to induce skin toxicity or whether treatment should be discontinued. Further prospective data are needed to establish the clinical value of skin toxicity as a predictive biomarker.

2.2.2 EGFR gene copy number

EGFR is localized on chromosome 7p11.2 which exhibits DNA copy number gain in approximately 35% of colorectal cancers [65]. Based on this observation, *EGFR* gene copy number has been investigated as a predictive biomarker for anti-EGFR therapy in multiple *post hoc* analyses. Study results have been aggregated in three meta-analyses [66-68], which broadly concurred in identifying gain of *EGFR* gene copy number as associated with improved outcomes among patients receiving cetuximab or panitumumab treatment. This association was found to be retained in subgroup analyses for patients with *KRAS* wild-type tumors, with one meta-analysis suggesting that this difference was not present in patients with *KRAS* mutated tumors [69]. However, the methodologies and criteria used for scoring increased EGFR gene copy number were highly inconsistent across different studies, and more research is required to clarify the predictive potential of this biomarker.

2.2.3 Amphiregulin (AREG) and epiregulin (EREG) expression

The EGFR ligands AREG and EREG are overexpressed in colorectal cancer at both the mRNA and protein levels [70, 71], and suppression of *AREG* or *EREG* gene expression reduces the therapeutic efficacy of cetuximab in tumor cell lines [72]. Accordingly, multiple studies have found evidence that the extent of expression of these ligands is related to efficacy of anti-EGFR therapy [71, 73–79].

For example, in the randomized phase III PICCOLO study of panitumumab and irinotecan *vs* irinotecan alone in fluorouracil-resistant mCRC, high messenger RNA (mRNA) expression of *EREG* or *AREG* (defined as either *EREG* or *AREG* in the top tertile for mRNA level) was a predictive marker for anti-EGFR therapy benefit in patients with wild-type *RAS* tumors. In contrast, patients with mutant *RAS* tumors gained no panitumumab therapy benefit regardless of ligand status [80]. Similarly, in the CO.17 study of cetuximab in chemotherapy-refractory mCRC, wild-type *KRAS* patients with high *EREG* gene expression obtained benefit from cetuximab therapy, while no benefit was observed in patients with low *EREG* expression; patients with mutant *KRAS* tumors showed no improvement on anti-EGFR therapy irrespective of *EREG* expression levels [76]. A retrospective analysis of the single-arm phase II NCT 00508404 study of first-line panitumumab plus FOLFIRI similarly also found a higher overall response rate for patients with wild-type *RAS* tumors and high *vs* low *AREG* expression [81]. A meta-analysis including eight studies that used anti-EGFR therapy alone or in combination with chemotherapy reported that *AREG*/*EREG* mRNA overexpression was associated with longer overall survival in patients with wild-type *RAS* tumors who received cetuximab or panitumumab treatment; *AREG* overexpression was further associated with longer PFS. In contrast, *AREG* and *EREG* was found not to have predictive value in patients with mutant *KRAS* tumors [82]. Given these encouraging data, further examination of these ligands in prospective controlled trials appears warranted.

2.2.4 BRAF mutation

The *BRAF* gene encodes a serine-threonine protein kinase that is an integral member of the RAS/MAPK signaling pathway. Approximately 10% of colorectal cancers harbor activating mutations in *BRAF*, with a valine (V) to a glutamic acid (E) substitution at codon 600 (V600E) accounting for more than 95% of alterations [16]. Mutations in *BRAF* are mutually exclusive with *KRAS* mutations in CRC [83]. Patients with mCRC who possess a *BRAF* mutation have significantly poorer prognosis as measured by PFS and OS, and mutational analysis is recommended

for prognostic stratification in guidelines from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology [84]. The relatively low mutation prevalence and strong association with prognosis in the metastatic setting have hampered conclusive evaluation of *BRAF* status as a predictive biomarker for anti-EGFR therapy in individual trials.

Two meta-analyses of randomized studies of anti-EGFR antibodies have been conducted with inconsistent findings. The first meta-analysis of eight randomized controlled trials published in seven studies concluded that there was insufficient evidence to demonstrate that mCRC patients with wild-type *RAS*/mutant *BRAF* tumors attain a different treatment benefit from anti-EGFR therapy as compared to patients with wild-type *RAS*/wild-type *BRAF* tumors [85]. However, the second meta-analysis of 10 randomized controlled trials from nine reports focusing on wild-type *RAS*/mutant *BRAF* tumors reported that anti-EGFR therapy provided no benefit in these patients, indicating presence of mutation as a marker of drug resistance. Based on these uncertain data, current guidelines for the treatment of mCRC do not recommend *BRAF* mutations as a biomarker for response to anti-EGFR therapy [84].

2.2.5 PIK3CA mutation

Phosphoinositide 3-kinases (PI3K) are a family of heterodimeric lipid kinases which consist of regulatory (p85) and catalytic (p110) subunits. PI3K is a key signaling mediator downstream of EGFR involved in the regulation of cell metabolism, growth, proliferation and survival. The *PIK3CA* gene encodes the catalytic subunit, p110α, which, when mutated in cancer, results in constitutively active PI3K signaling. *PIK3CA* mutations are present in approximately 10–20% of colorectal cancers, with missense mutations in exon 9 (helical domain) and exon 20 (kinase domain) being the most common alterations [86, 87]. Notably, biochemical studies comparing mutant p110α proteins have established that exon 9 and exon 20 substitutions have different mechanisms of action. Exon 9-mutant p110α protein induces cell transformation independently of binding to p85 but requires interaction with RAS-GTP, whereas exon 20-mutant p110α protein is active in the absence of RAS-GTP binding but is dependent on the interaction with p85 [88].

PIK3CA mutations have been investigated as a potential predictor of anti-EGFR therapy efficacy, with studies considering mutation status overall or for exons 9 and 20 separately. Again, conclusive analyses from individual studies have been hampered by the relatively low mutation prevalence, with *PIK3CA* mutations tending to co-occur with *KRAS* mutations [87]. A series of meta-analyses have been conducted to consolidate findings, indicating that *PIK3CA* mutations as a whole are associated with a lack of anti-EGFR therapy response in patients with wild-type *RAS* tumors [89–93]. Some studies further suggest that the predictive power may be confined to exon 20 mutations, although sample size remains limited [90, 91, 94]. However, these meta-analyses have included many of the same studies, as well as observed and acknowledged between-study heterogeneity. Further investigations are needed before definitive conclusions regarding the predictive value of *PIK3CA* mutations for clinical decision making can be drawn, and *PIK3CA* mutational analysis of colorectal carcinoma tissue for therapy selection outside of a clinical trial is currently not recommended [84].

2.2.6 PTEN loss

PTEN is a negative regulator of the PI3K/AKT pathway downstream of EGFR through its lipid phosphatase activity. PTEN is a tumor suppressor gene

in colorectal cancer, with inactivating mutations or loss of protein expression observed in approximately 5% and 30% of sporadic colorectal cancers [87, 95, 96].

With respect to response to anti-EGFR therapy, a number of studies have indicated an association with PTEN loss and lack of response to cetuximab and panitumumab [97–100], although other reports have not identified this relationship [101–103]. There are also data to suggest that some discordance in PTEN protein expression may exist between primary tumors and metastases [104]. Several meta-analyses have considered published findings, supporting the notion that loss of PTEN protein expression and/or mutation are predictive of worse outcomes in patients with wild-type *KRAS* tumors treated with anti-EGFR therapy [91, 92, 105]. However, given a high level of variability in methods for assessment of PTEN expression between studies, including IHC scoring algorithms, and the potential inconsistency in expression between primary and metastatic tumor samples, loss of PTEN expression cannot yet be regarded as a reliable predictive biomarker. Further investigation and prospective large randomized clinical trials are still required to fully confirm the role of PTEN in anti-EGFR therapy resistance.

3. Conclusion

The introduction of multiple chemotherapy and biological therapy options for the treatment of CRC over the past few decades have driven an increased need for predictive biomarkers to select the most appropriate therapy for each patient. Biomarker guided treatment selection is critical to improving patient outcomes, reducing exposure to ineffective lines of treatment that are associated with significant toxicities and costs. For the use of the anti-EGFR antibodies cetuximab and panitumumab, current best clinical practice mandates that assessment of all common mutations in *KRAS* and *NRAS* be undertaken at the time of diagnosis of mCRC. Sidedness is also an important factor and it is recommended to limit anti-EGFR therapy to cases with left-sided primary tumor location [53].

Mutations and amplifications of several genes other than *RAS* have been investigated as potential predictive biomarkers of response to anti-EGFR therapy. These include *EGFR* gene copy number, *BRAF* and *PIK3CA* mutation as well as PTEN loss (mutation and loss of protein expression). The individual frequencies of all of these mutations and amplifications are low and methodologies to determine DNA copy number or protein expression have been highly variable across studies, thus whether these alterations are true biomarkers for anti-EGFR therapy resistance remains uncertain. Expression of the EGFR ligands AREG and EREG are an interesting avenue to explore, but current evidence is insufficient to recommend routine testing in clinical practice. Skin toxicity is a potential predictive marker in wild-type *RAS* patients receiving anti-EGFR therapy, but prospective randomized data are required to demonstrate clinical utility and determine how this information is best used to inform patient management (dose escalation *vs* treatment discontinuation).

While significant progress has been made in identifying predictive biomarkers for anti-EGFR therapy, with *RAS* mutation status and tumor sidedness endorsed as clinical diagnostics, many patients treated with cetuximab and panitumumab as selected by these parameters still do not experience treatment benefit. Further basic biology and clinical studies are clearly warranted to improve our understanding of EGFR signaling to identify novel biomarkers predictive of anti-EGFR therapy response and to develop more refined companion diagnostics.

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Conflict of interest

The authors declare no conflict of interest.

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