Targeting EGFR in metastatic colorectal cancer beyond the limitations of KRAS status: alternative biomarkers and therapeutic strategies

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Summary

Patients with metastatic colorectal cancer (mCRC) have a very poor prognosis. Incorporation of targeted molecular therapies, such as the anti-EGFR monoclonal antibodies Cetuximab and Panitumumab, into treatment regimens have improved outcomes for patients with wild-type RAS tumors. Yet, response rates remain low and overall survival times are short. Increased understanding of oncogenic signaling pathways within the tumor, and how these are regulated by the inflammatory tumor microenvironment, is a priority to facilitate the development of biomarkers to better guide the use of existing therapies and to develop new ones. Here, we review recent pre-clinical and clinical progress in the development of biomarkers for predicting response to anti-EGFR therapy in mCRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both men and women, ranking among the top 4 leading causes of cancer-related deaths worldwide (accounting for more than 10% of total cancer deaths in Europe) [1]. Early-diagnosed and localized CRC, that is stage I-II according to the American Joint Committee on Cancer (AJCC) classification system [2], is successfully treated with surgery, with or without adjuvant chemotherapy, and generally has 5-years survival rates higher than 90%. However, a large proportion of newly diagnosed CRCs are stage IV metastatic cancers (mCRC), for which 5-years survival rates are less than 12% [3,4]. Patients with mCRC may receive chemotherapy pre-surgery to downsize tumors, following surgery or palliatively. The chemotherapeutic drugs most frequently used are 5-Fluorouracil given in combination with Leucovorin and either Oxaliplatin or Irinotecan in the FOLFOX or FOLFIRI regimens, respectively. These regimens can be further combined with biologic molecular targeted therapies such as monoclonal antibodies (mAbs) specific for the epidermal growth factor receptor (EGFR), Cetuximab (Erbitux®, Merck Serono, a chimeric IgG_1 mAb) and Panitumumab (Vectibix®, Amgen, a humanized IgG_2 mAb) [5]. These antibodies bind to the extracellular domain of EGFR, inhibiting its auto-phosphorylation and subsequent activation of oncogenic downstream signaling pathways. Several trials have shown clinical efficacy of Cetuximab in mCRC patients that have become refractory to standard treatment (reviewed in [5] and [6]). However, in un-selected patient cohorts the response rate to Cetuximab is less than 20%. The efficacy of Cetuximab and Panitumumab is not specifically related to EGFR protein levels, gene copy number, or molecular alterations [5,7], as somatic mutations in EGFR are a rare event in CRC. Therefore, efforts to evaluate possible biomarkers of response shifted rapidly to the signaling pathways downstream of EGFR, and led to the seminal finding that *KRAS* mutation status is highly predictive of Cetuximab [8] or Panitumumab [9] resistance. Consequently, anti-EGFR therapy is now only recommended for patients that have wt-*KRAS* mCRC. However, even in this context, only up to 50% of patients respond to EGFR mAbs, and responders eventually develop resistance [5].

The search for more accurate biomarkers of clinical response to anti-EGFR mAbs is ongoing with the majority of putative targets being investigated having an impact on the EGFR signaling axis. In this review we will highlight novel alternative strategies to target the EGFR pathway in mCRC, reporting on recent pre-clinical and clinical developments in biomarker discovery, and their implications for targeted therapy.

Targeting molecular pathways downstream of EGFR

The two main molecular signaling pathways activated downstream of EGFR are dependent on RAS-RAF-MAPK and PI3K-AKT-mTOR [10]. Both of these are crucially involved in regulating cellular events promoting a tumorigenic phenotype (such as cell growth, differentiation, migration and apoptosis, as summarized in Figure 1).

The RAS-RAF-MAPK pathway

The *RAS* family of oncogenes (including the highly homologous *KRAS*, *NRAS* and *HRAS*) encodes guanosine di/tri-phosphate binding proteins that act as mitogenic signal transducers of the MAPK signaling pathway. RAS is activated or inactivated by selective binding of GDP/GTP, which is mediated by the Grb2/SOS complex bound to tyrosine-phosphorylated EGFR. Once activated, RAS phosphorylates BRAF, activating a sequential cascade leading to MAPK-mediated regulation of transcription factors and modulation of gene expression [11].

KRAS

KRAS is commonly mutated in CRC. Somatic point mutations are present in 35-45% of mCRC [5], and mainly occur in codon 12 and 13 [7]. The presence of a *KRAS* mutation in mCRC is established as a predictive biomarker for resistance to EGFR-targeted therapy. This was evidenced in initial retrospective analyses of small cohorts [8,12,13] and then confirmed in large randomized phase II/III clinical trials [14,15] where the benefits of adding Cetuximab to chemotherapy were only seen in wild-type KRAS patients. The pivotal phase III trial comparing Panitumumab monotherapy to best supportive care [9] showed that also for Panitumumab the efficacy is confined to patients with wt-KRAS tumors. This was subsequently confirmed in two phase III trials comparing panitumumab treatment addition to chemotherapy [16,17]. Consequently, anti-EGFR mAbs are only recommended for

patients with wt-*KRAS* tumors [18]. Interestingly, different *KRAS* mutations might confer different degrees of resistance to Cetuximab or Panitumumab, as suggested by *in vitro* analysis of mutant cell lines [19] and retrospective analysis of clinical studies [20,21]. Specifically, the KRAS codon 13 glycine-to-aspartate mutation (p.G13D) could be associated with increased response to anti-EGFR mAbs [19,20], while generally alternative KRAS mutations in codon 13, 61 or 146 appear to be associated with resistance to anti-EGFR therapy [21-23], and therefore could be used to further refine selection of candidate patients for targeted therapy. This hypothesis however is based solely on retrospective data and would need to be confirmed in prospective trials.

NRAS

Mutations in *NRAS* are very rare in mCRC (between 1 and 3%), and are ususally mutually exclusive with *KRAS* mutations. However, De Roock and colleagues demonstrated a strong association between mutations in *NRAS* and a reduced response rate to anti-EGFR therapy [24]. This was recently confirmed in a retrospective analysis of 430 wt-*KRAS* patients from the CRYSTAL trial, where the presence of mutations in *NRAS* or in *KRAS* (additional mutations other than those in codon 12 or 13) completely abrogated the efficacy of Cetuximab treatment [25]. The predictive role of *NRAS* mutations was also confirmed for Panitumumab in retrospective analyses of two phase III trials comparing Panitumumab in addition to either FOLFIRI [16,26] or FOLFOX4 [23] regimens. In the study of Douillard and colleagues, mutations in *KRAS* exon 3 and 4 (codons 61,117,146), *NRAS* exon 2 and 3 (codons 61,117,146), and BRAF exon 15 (codon 600) were analyzed together, and the predictive role of any RAS mutation (other than *KRAS* exon 2 mutations) was reported [23]. In an analysis of 786 patients the *NRAS* mutation rate was 6% in the un-selected population, but 15% in selected wt-*KRAS*/wt-*BRAF* patients [27]. Patients with tumors harboring *NRAS* mutations had a significantly shorter OS in comparison to wild-type patients, with a median OS of 25.6 months and 42.7 months, respectively. Furthermore, the prognostic impact of *NRAS* mutation was confirmed in multivariate analysis (HR = 1.75, 95% CI 1.1.3–2.72; $p = 0.013$) [27]. As a consequence of this and other recently published data, testing for *NRAS* mutation

has been introduced into clinical practice. From December 2013 the European Commission has amended the product information for Cetuximab and Panitumumab, updating the indication for the treatment of patients from *KRAS* wild-type to a broader *RAS* wild-type mCRC.

BRAF

BRAF is a serine/threonine kinase which is directly activated by RAS and is mutated in a wide range of human malignancies, including CRC. The most prevalent mutation (the V600E substitution) renders BRAF constitutively active, therefore resulting in the same downstream effect induced by *RAS* mutations. As a consequence, mutations in *BRAF* and *KRAS* are generally mutually exclusive. The reported rate of *BRAF* mutations in CRC is 5-22%, and this is strongly associated with serrated polyps, microsatellite instability (MSI) and CpG island metylator phenotype (CIMP) [28]. The absence of a *BRAF* mutation increases the chance of clinical benefit from both Cetuximab and Panitumumab therapy [24,29]. However, further retrospective analysis of clinical trials have challenged these results, showing that the presence of a *BRAF* mutation is a negative prognostic indicator being associated with shorter OS independently of Cetuximab use [5,30]. Nevertheless, testing for *BRAF* mutations is becoming part of routine clinical management of mCRC patients in some centers.

Therapeutic targeting of the RAS pathway

Beside its role as a predictive biomarker, RAS has been also investigated as a putative therapeutic target in CRC. RAS inhibitors have shown potential clinical utility in animal models, e.g. Manumycin A reduced formation of aberrant crypt foci (pre-neoplastic lesions) in an azoxymethane-induced rat colorectal carcinogenesis model [31]. However, the clinical development of RAS inhibitors has not progressed until the recently announced initiative by the U.S. National Cancer Institute to fund a multi-institutional research project aimed at investigating novel approaches for pharmaceutical targeting of RAS in human cancer [32].

Pharmacological targeting of RAF with drugs such as PLX4032 (vemurafenib) are in clinical use for metastatic melanoma harboring the *BRAF* V600E mutation, but have only demonstrated modest clinical activity in a phase I trial of mCRC [33]. Pre-

clinical work on this inhibitor suggested it might have increased benefits in combination regimens with Akt, VEGF, or EGFR inhibitors, including Cetuximab [33,34], although this needs to be confirmed in clinical studies. A recent case report [35] of a patient with wt-*KRAS*, *BRAF*-mutant mCRC who was treated with off-label sorafenib (a multi-kinase inhibitor with activity against RAF) in combination with Cetuximab reported a mixed radiological response with a PFS of 7 months and minimal toxicity, suggesting that this may be a useful drug combination to pursue in patients using a personalized approach.

Inhibition of MEK downstream of RAS/RAF is another putative therapeutic avenue with potential clinical benefits, based on pre-clinical evidence suggesting that activation of MEK and the downstream MAPK signaling is involved in both primary and acquired resistance to anti-EGFR therapy [36]. The MEK inhibitor Selumetinib (AZD6244) was examined in a phase II trial in combination with Irinotecan for patients progressing on first-line chemotherapy. Although terminated early, this study showed promising results in terms of response rate and median progression-free survival [37]. A synthetic-lethal interaction screening showed that a combination regimen of MEK and RAF inhibition was efficient at inducing sustained and prolonged inhibition of the MAPK signaling and overcoming resistance to Selumetinib [38].

The PI3K-Akt-mTOR pathway

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signals downstream of EGFR to promote cancer growth, survival and metabolism. PI3K is activated directly by RAS or by receptor tyrosine kinases, mainly through the Akt-mTOR pathway (Figure 1) [39]. Somatic mutations in the *PIK3CA* gene, encoding for the p110 catalytic subunit of PI3K, are common in different types of epithelial malignancies, and are reported in 15-20% of CRC [8].

The prognostic and predictive role of these activating mutations has been extensively studied in CRC but the evidence remains rather conflicting [5,40,41]. However, a recent meta-analysis including 693 patients concluded that there was an

association between *PIK3CA* mutations and a reduced objective response to Cetuximab or Panitumumab in wt *KRAS* patients (OR = 0.42; 95% CI 0.23–0.75; p = 0.003) [42]. The lack of consensus could be primarily due to the fact that different *PIK3CA* mutations (in exon 9 or 20) can have a different impact on the EGFR-mediated signaling [43] and therefore have different oncogenic properties that may impact the response to anti-EGFR therapy [24]. Further standardization of the methods used to classify *PIK3CA* status may lead to a consensus on its clinical usefulness as a biomarker in CRC.

The PI3K pathway can also be activated by mutations or epigenetic inactivation of the phosphatase PTEN, which directly inactivates PI3K. The prevalence of PTEN inactivation in CRC is higher than that of *PIK3CA* mutations, however evidence of its prognostic role is even more conflicting primarily due to the lack of standardization in the methods used for testing PTEN expression by immunohistochemistry [44]. Another possible confounding factor is that *PIK3CA* mutations or PTEN inactivation can occur concomitantly with *KRAS* or *BRAF* mutations, therefore masking their individual prognostic significance [5]. In fact, significant correlation between the occurrence of *RAS* and *PI3K* mutations was found in one third of the 224 CRC samples analyzed in the TCGA cohort [45], and a strong association was also reported in a cohort of 238 mCRC patients [46]. The overlapping pattern of expression of these molecular alterations supports the use of multiple biomarkers as opposed to studying each individual marker as a separate biological event.

Use of multiple biomarkers and acquired resistance

The idea that using multiple biomarker simultaneously could lead to better patient stratification is further supported by a retrospective analysis of 132 mCRC patients treated with Cetuximab or Panitumumab [47]: when the expression of PTEN and *KRAS*, *BRAF* and *PIK3CA* mutations were considered concomitantly, up to 70% of patients unlikely to respond to anti-EGFR therapies could be identified. The probability of response was high among patients with no alterations (defined as *quadruple negative* subgroup), low in patients with 1 alteration, and absent in patients with 2 or more alterations [47]. Furthermore, a recent meta-analysis

including 1773 patients confirmed the increased predictive value of combined biomarkers including *PIK3CA*, *PTEN* and *BRAF* alongside *KRAS* mutations [48]. A gene expression signature combining perturbations in *KRAS*, *BRAF* and *PIK3CA* was recently developed and used to identify patients with an activated EGFR pathway caused by any activation mechanism. This gene signature was strongly associated with lack of response to Cetuximab (HR=2.51, p=0.0009 for combined wt patients versus *oncogenic activated* patients) [49]. Therefore, developing robust techniques to detect multiple mutations in genes downstream of EGFR has the potential to improve the accuracy of patient selection for anti-EGFR mAb therapy and subsequently increase the response rate.

Furthermore, tumors that are initially wt-KRAS can develop other mutations and subsequently acquire resistance to anti-EGFR therapies. For example, Misale and colleagues analyzed tumor biopsies from 10 CRC patients who had progressed after treatment with either Cetuximab or Panitumumab: they reported additional KRAS mutations or amplification in 7 out 10 patients [50]. Similarly, Diaz and colleagues analyzed serial serum samples from chemorefractory mCRC receiving single-agent therapy with Panitumumab who had progressed over the course of treatment. They found 9 out of 28 patients (38%) developed detectable mutations in KRAS in their sera during the course of treatment, three of which developed multiple different KRAS mutations [51].

Targeting multiple biomarkers will be critical when it comes to designing optimal combinatorial regimens of kinase inhibitors which are showing promise [52]. Garrido-Laguna and colleagues reported a complete or partial response in 23% of 80 mCRC patients treated with PI3K/Akt/mTOR inhibitors, with stable disease of 6 months or more. Interestingly, the response was independent of the presence of a *PIK3CA* mutation [46]. Such combination regimes have the added advantage that they may overcome the intrinsic resistance of tumors with *KRAS* mutations to EGFR inhibition [53]. The multi-kinase inhibitor dasatinib (BMS-354825, targeting the Src family of kinases) in combination with Cetuximab showed anti-proliferative effects in mutant KRAS CRC cell lines *in vitro* and in corresponding xenografts [54].

Recently, a molecularly stratified and adaptive randomized trial has been initiated in the U.K. for patients with advanced mCRC. The FOCUS4 trial will use a multi-arm, multi-stage design to assign patients to the most appropriate study arm based on the molecular characterization of their tumor, to assess the efficacy of different combination regimens of kinase inhibitors against placebo (http://www.focus4trial.org). A number of such strategies have also begun early clinical studies (summarized in Table 1). The results of these studies are eagerly awaited and will provide crucial information for the design of future trials of combination chemotherapy in mCRC.

Targeting alternative growth factor receptors

In addition to EGFR there are 3 additional members of the ErbB family of receptors, HER2, HER3 and HER4. Compensatory mechanisms between members of the ErbB family lead to aberrant activation of receptor tyrosine kinases (RTK) in the context of *de novo* and acquired resistance to RTK inhibition [55] and this should be considered when targeting this pathway.

Alterations (either mutations or amplifications) of at least one of the four ErbB family members are present in 13-53% of CRC cases, being higher in those tumors that present a high mutation rate [45]. Bertotti and colleagues described *HER2* amplification in clinically un-responsive wt-*KRAS* mCRC, suggesting that *HER2* amplification is a negative determinant of response to anti-EGFR antibodies in mCRC tumors that do not harbor genetic alterations of the RAS pathway [56]. Interestingly, in a group of selected Cetuximab-resistant tumors, *HER2* amplification was seen in 36% of cases [56], suggesting the possibility of using HER2 as a biomarker in patients who have relapsed following EGFR-targeted therapy. The work of Martin et al. also confirmed that *HER2* gene copy number status may influence the response to either Cetuximab or Panitumumab in mCRC patients [57]. Further work is needed to develop therapeutic regimens that might prove beneficial to treat *HER2*-amplified mCRC that is resistant to anti-EGFR therapy. Recently, a single-arm, open-label, multicenter phase I/II study was designed to assess the safety and efficacy of Pertuzumab and Cetuximab in patients with Cetuximab-

resistant wt-*KRAS* mCRC. The combination was associated with potential antitumor activity, but it was not tolerable due to overlapping toxicities, and the trial was terminated early [58].

HER3 has also been implicated in CRC, with expression independently associated with worse clinical outcome [59]. Interestingly, HER3 down-regulation by RNA interference abrogated cell proliferation, migration, and invasion in four different CRC cell lines [60]. Furthermore, suppression of HER3 could sensitized *KRAS* mutant colon cancer cells to MEK inhibitors [61].

The *MET* gene, which encodes the receptor for Hepatocyte Growth Factor (HGF), has an oncogenic role in several human tumors. Over-activation of MET signaling has been implicated in both acquired and *de novo* resistance to anti-EGFR therapies using *in vitro* and *ex vivo* models of wt-*KRAS* mCRC [62]. MET signaling is particularly active in a sub-population of stem-like cancer initiating cells, counteracting the effects of EGFR inhibition through cytokines (including HGF) secreted by cancer-associated fibroblasts [63]. Activation of MET and downstream JAK1/2-STAT3 signaling is also responsible for acquired resistance to MEK inhibition in mut-*KRAS* CRC. Pharmacological blockade of this resistance pathway with either c-MET or JAK1/2 inhibitors synergistically increased MEK-inhibitor-induced apoptosis and growth inhibition *in vitro* and *in vivo* in mut-*KRAS* models [64]. Given the partial redundancy of the signal originating from EGFR and other receptors members of the ErbB family, it is possible to speculate that these receptors will add predictive information by indicating network rewiring characteristic of resistance to therapy.

Targeting ErbB ligand expression upstream of EGFR

A diverse family of ligands (EGF, HB-EGF, TGF-α, amphiregulin, epiregulin, neuregulin) can bind to the four members of the EGFR family (EGFR, HER2, HER3, HER4). Each ligand can bind one or multiple ErbBs, determining the formation of specific receptor homo- or hetero-dimers, and consequently influencing the type, strength and duration of the downstream signaling [55].

ErbB ligands have been investigated as putative predictive biomarkers in mCRC.

Patients whose tumors express high levels of the EGFR ligands epiregulin (EREG) and amphiregulin (AREG) are more likely to respond to Cetuximab and high levels of AREG and EREG was also associated with a 50% increase in both PFS and OS [65]. This was later confirmed in a secondary analysis of samples derived from a phase I dose-escalation study of first-line Cetuximab combined with an Irinotecan-based regimen [66]. Interestingly, *TGFA* (the gene encoding for TGF-α,) demonstrated a reciprocal expression pattern, with low expression being associated with best clinical response to Cetuximab. More recently, Pentheroudakis and colleagues have shown that high mRNA levels of AREG are predictive of longer survival in patients with wt-*KRAS*, but not in *KRAS* mutant tumors. In contrast, high mRNA levels of EREG were associated with favourable prognosis irrespective of *KRAS* mutational status [67]. This was also confirmed in a phase III clinical trial of Cetuximab plus best supportive care (BSC) vs BSC alone, where patients with high *EREG* expression appeared to benefit more from Cetuximab therapy compared with low expression only in pre-treated wt-*KRAS* group [68]. Altogether this data suggests that monitoring of EGFR ligand expression may be of clinical use in the management of mCRC and warrants further validation. However, for this to happen, a consensus needs to be reached on the exact threshold levels and subsequent definition of *over-expression* for candidate biomarkers measured by mRNA expression analysis.

The release and activation of ErbB ligands is catalysed by 2 matrix metalloproteinases of the ADAMs (a disintegrin and metalloproteinase) family, ADAM-10 and ADAM-17 [69]. Because of the predictive role of EGFR ligands, these enzymes have been investigated for their putative prognostic and/or predictive role. ADAM-17 is overexpressed in primary and mCRC tumors compared with normal colonic mucosa [70], and combining an EGFR mAb with an ADAM-17 inhibitor *in vitro* resulted in cooperative growth inhibition in colorectal cancer cells [70]. The group of Van Schaeybroeck demonstrated that ADAM-17 activity may contribute to the resistance to chemotherapy via oncogenic KRAS signaling. Mutant *KRAS* CRC tumors were vulnerable to MEK1/2 inhibitors, at least in part, due to their dependency on ADAM-17 [71,72].

Aside from its putative role as a predictive biomarker, ADAM-17 could be targeted in combination with EGFR antibodies or other kinase inhibitors, as inhibitors of ADAM-17 have already shown promising results in pre-clinical models of breast [73] and ovarian [74] cancers, as well as inhibiting EGFR-mediated inflammatory signaling in the chronic autoimmune disorder Sjögren's syndrome [75]. As EGFR ligands are capable of activating paracrine proliferative signaling in neighboring cells, and because ADAM-17 is also involved in shedding of chemokines from immune cells [76], measuring EGFR ligands in combination with the mutation status of the primary tumor could be of prognostic or predictive value. Integrating information regarding mutational status of the tumor with its microenvironment would help to define the mechanisms of resistance and immune escape from Cetuximab therapy.

Role of ADCC and interaction with immune effector cells

In addition to blocking EGFR- mediated signaling, anti-EGFR mAbs also exert therapeutic effects via antibody mediated cellular cytotoxicity (ADCC). Upon the binding to EGFR, the Fc region of the antibody remains exposed, where it can be recognized by immune effector cells via their FcγR (Fragment c Gamma Receptors). Subsequently, the opsonized tumor cells are phagocytosed or lysed through ADCC by natural killer cells, macrophages and other immune effector cells bearing the FcγR. Considering that IgG1 binds most effectively to FcγR compared to other IgG isotypes, Cetuximab is more likely to stimulate ADCC efficiently than Panitumumab (IgG2). The ADCC activity of Cetuximab is dependent on appropriate glycosylation of the antibody and tumor cell EGFR expression [77]. The correct dosage of Cetuximab and the presence of tumor infiltrating $CD8^+$ T cells are also key determinants of successful triggering of ADCC [78]. Calemma and colleagues have addressed the hypothesis that variants of human IgG-receptors could influence the extent of ADCC and, thus, response to anti-EGFR therapy. They demonstrated that homozygous carriers of the 158V allele of the FcγRIIIa show a high response rate to Cetuximab and a significantly improved prognosis in wt-*KRAS* mCRC [79]. ADCC could be also responsible for the clinical response to anti-EGFR antibodies observed

in *KRAS* mutant tumors. In fact, higher EGFR expression can predict susceptibility to Cetuximab-induced immune killing of CRC cells occurring independently of mutation in either *KRAS*, *BRAF* or *PIK3CA* [80]. Strategies to improve Cetuximab-induced ADCC are being investigated; a sequential approach with 2 different mAbs have shown promising results in *in vivo* models of $EGFR^+$ tumors: first Cetuximab activates surrounding NK cells via the Fc-FcγR interaction, thereby increasing NK cell expression of co-stimulatory markers, such as CD137. An agonistic mAb against CD137 is then administered to enhance the activated NK cell's ability to kill Cetuximab-coated tumor cells [81]. Co-administration of TLR3 agonists increases Cetuximab-mediated ADCC against head and neck cancer cells, as well as dendritic cell maturation and cross-priming of EGFR-specific $CD8^+$ T cells [82]. So strategies that target immune effectors to enhance ADCC may enhance the efficacy of anti-EGFR therapies.

mCRC patients treated with anti-EGFR mAb often develop dose-limiting skin rashes. Interestingly, the severity of cutaneous side effects correlates with an increased response rate to the mAb [83]. Furthermore, mCRC patients treated with Cetuximab plus chemotherapy showed altered cytokine production by peripheral blood cells after treatment (specifically, an increase in IL-2, IFN-γ, IL-12 and IL-18, and a decrease in IL-4 and IL-10) which correlated with response to therapy [84], thus suggesting that monitoring of the peripheral immune system function could be used as surrogate marker in predicting treatment-related outcome in these patients. Furthermore EGFR inhibition may activate the programmed cell death protein 1 (PD-1/PD-L1) pathway to alter immune cell function. This highlights possible therapeutic opportunities stemming from the interplay between cancer cells and the immune system; for example, PD-1 antibody blockade improved the survival of mice with EGFR-driven lung adenocarcinomas by enhancing effector T-cell function and lowering the levels of tumor-promoting cytokines [85].

Role of miRNA in resistance to EGFR-targeted agents

Micro RNAs (miRNAs) control the post-transcriptional regulation of gene expression by directly binding to the 3'UTR sequence of their target genes, resulting in either suppression of translation or degradation of mRNA from protein-coding genes [86]. This mechanism have been implicated in different patophysiological conditions including cancer, and several miRNAs display dysregulated expression patterns in CRC [87,88]. Among these, miR-143 and miR-145 are perhaps the most widely studied as they are commonly down-regulated in a wide range of cancers and coordinate tumor-suppressive functions. Both miR-143 and miR-145 directly target and down-regulate expression of KRAS [89], as well as other genes involved in the EGFR signaling pathway. Pichler and colleagues analyzed 77 wt-KRAS tumors treated with Cetuximab or Panitumumab, and they found down-regulation of miR-143 in 61% of the samples; low levels of miRNA-143 expression are an independent negative prognostic factor, being associated with reduced cancer-specific survival. However, no significant association was found with response to EGFR-targeted therapy [90]. In addition, overexpression of either miR-143 or miR-145 in HCT116 cells abrogates signaling through the MAPK, PI3K and c-Jun NH2-terminal kinase (JNK) pathways by down-regulation of *KRAS* in addition to down-regulation of a cohort of genes in the MAPK signaling cascade [91]. A lack of miR-143 and miR-145 expression has been demonstrated to be a frequent feature of colorectal tumors, however a recent paper questioned the presence of these miRNAs in colorectal epithelial cells, showing with the use of mouse models that both miR-143 and miR-145 are almost exclusively expressed in mesenchymal cells (mainly fibroblasts and smooth muscle cells) of the intestine, suggesting that the tumor-suppressive role of the two miRNAs might be exerted through the tumor microenvironment [92]. Recently, miR-143 was associated with reduced overall survival in 138 patients with mCRC in 3rd line therapy with Cetuximab and irinotecan in a prospective phase II study [93]. This study identified another miRNA, miR-345, as being a strong prognostic and predictive marker associated with low OS and PFS in multivariate analysis, as well as with lack of response to Cetuximab [93]. Using both retrospectively and prospectively collected fresh-frozen and FFPE samples from chemorefractory mCRC treated with anti-EGFR antibodies, miR-31-3p was also

identified as a new mCRC biomarker whose expression level allows for the identification of patients with wild-type *KRAS* mCRC who are more likely to respond to anti-EGFR therapy [94]. The direct targeting of non-coding RNAs that participate in cancer pathogenesis is a possible therapeutic options that is being explored, with agents like antisense oligonucleotides, miRNA mimics, anti-miRNA, and antagomirs currently being tested in pre-clinical and clinical studies [87]. Therapeutic targeting of non-coding RNAs has the potential advantage of inhibiting multiple pathways simultaneously, since every miRNA can regulate multiple targets; the challenge in the immediate future will be to achieve efficient and safe systemic delivery of therapeutic miRNAs *in vivo* [95].

Conclusions and Future Perspectives

In the relatively short time since the approval of the anti-EGFR mAbs, Cetuximab and Panitumumab for the treatment of mCRC, research into companion predictive biomarkers has progressed at a good pace. Although only *RAS* mutations are currently clinically used as a predictive tool for patient selection, many other candidate biomarkers are in the pipeline at different stages of pre-clinical and clinical development. Together with *RAS* mutation status, these novel markers can predict for primary resistance to anti-EGFR targeted therapy and account for up to 80% of the unresponsive cases [5]. This suggests that there certainly are additional, yet to be discovered biomarkers of resistance, and significant challenges still lie ahead in this field. Furthermore, with the only exception of BRAF, for the majority of the reported biomarkers the data available is still at the proof-of-principle stage, and significant efforts will be required to address the need of an adequate prospective validation of these markers which would improve appropriate selection of patients for treatment.

The current fast-paced development of technologies such as Next Generation Sequencing (NGS) will soon allow for their routine use in clinical practice to type the mutational status of each tumor. This detailed molecular classification of tumors will allow selection of the most appropriate treatment from an array of specific inhibitors. Whole genome sequencing of CRC samples has already furthered our

knowledge of the mutational landscape of CRC [45], and applications of NGS are becoming a standard in the management of CRC for high-throughput detection of multiple predictive mutations following the new international guidelines [96], effectively delivering personalized medicine. Future applications of NGS will allow for better tools for early detection of both familiar and sporadic CRC, and personalization of treatment to specific patient tolerances (pharmacogenomics) [97]. Concerted efforts in the field of systems biology will help to extract biological meaning from this vast amount of data to further our understanding of biological pathways interaction, and to design treatment strategies based on drug combinations that will optimize response and overcome resistance. The interaction between the tumor and its immune microenvironment especially holds promising therapeutic avenues to be explored. Cetuximab alters the immune response in some patients, and a better understanding of the mechanisms involved will allow the drug to be used in combination with immune therapies, an opportunity that is still at the early stages but that will surely see substantial improvements in the next five to ten years.

Executive summary

Introduction

- Anti-EGFR monoclonal antibodies are recommended for patients with wild-type *RAS* metastatic CRC, but have response rates of 40% in selected population.
- Alternative predictive and prognostic biomarkers are needed to improve patient outcomes.

Targeting molecular pathways downstream of EGFR

- Somatic mutations in *KRAS* are present in 35-45% of mCRC and are established as a predictive biomarker for resistance to EGFR-targeted therapy.
- Mutations in *NRAS* and *BRAF* are less frequent but have predictive significance in retrospective analysis.
- Mutations in *PIK3CA* or PTEN expression may also be predictive but the evidence is more contradictory and technical improvements in detection methods are needed.
- The predictive value of combined multiple biomarkers has been confirmed in large meta-analysis.

Targeting alternative growth factor receptors

- HER2 amplification is associated with wt-RAS tumors un-responsive to Cetuximab/Panitumumab, although the prevalence might be as low as 2%.
- HER3 over-expression is found in up to 70% of tumors and is an independent prognostic marker with therapeutic potential in pre-clinical studies.
- Amplification and over-activation of MET is involved in both primary and secondary resistance to anti-EGFR therapy.

Targeting ErbB ligand expression upstream of EGFR

- High levels of EGFR ligands EREG and AREG, and low levels of TGF-α are predictive of response to anti-EGFR mAbs, although this might be restricted to wt-RAS patients.

- ADAM-17 is involved in EGFR ligand shedding and in resistance to chemotherapy in mut-RAS tumors. ADAM-17 inhibition has potential benefits in combination regimens.

Role of ADCC and interaction with immune effector cells

- Increased activation of ADCC responses improve prognosis for wt-RAS patients and can lead to partial response in mut-RAS tumors.
- Response to EGFR therapy is associated with the severity of dermatological side effects, although with unknown mechanisms.
- Impaired cytokine production in peripheral blood mononuclear cells correlates with response to therapy.

Role of miRNA in resistance to EGFR-targeted agents

- Several miRNAs, including miR-143, miR-145, miR-345 and miR-31-3p, are differentially regulated in CRC and are prognostic factors, but their predictive value is controversial, and their therapeutic potential is still unclear.

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Table 1. Currently active clinical trials of biological agents for selected targets in combination with EGFR monoclonal antibodies (source: clinicaltrials.gov, accessed august 2014). Only active/ongoing trials are reported, with their recruiting status indicated (Yes/No)

Target	Compound	Clinical Trial ID	Description	Phase	Recruiting	Sponsor
BRAF	dabrafenib	NCT01750918	combination with MEK inhibitor (GSK2141795) + Panitumumab	1/2	Y	GlaxoSmithKline
	LGX818	NCT01719380	combination with PI3K inhibitor (BYL719) + Cetuximab	1b/2	Y	Novartis
	BAY 43-9006	NCT00326495	dual BRAF/VEGF inhibition in combination with Cetuximab	1/2	Y	NCI
PI3K-mTOR	PF-05212384	NCT01925274	combination with Irinotecan + Cetuximab	2	Y	Pfizer
	PX-866	NCT01252628	combination with Cetuximab	2	N	Oncothyreon
MEK 1/2	MEK162	NCT01927341	combination with Panitumumab	1b/2	Y	Novartis
HER2	neratinib	NCT01960023	combination with Cetuximab	1/2	Y	National Surgical Adjuvant Breast and Bowel Project
c-MET	INC280	NCT02205398	combination with Cetuximab	1b	N	Novartis
	Tivantinib	NCT01892527	combination with Cetuximab	2	Y	Istituto Clinico Humanitas
Immunotherapy	EGFRBi armed ATC	NCT01420874	Anti-CD3, anti-Cetuximab Armed Activated T Cells	1b	Y	Barbara Ann Karmanos Cancer Institute
	DCVax-Direct	NCT01882946	Autologous, activated dendritic cells for intratumoral injection	1/2	Y	Northwest Biotherapeutics

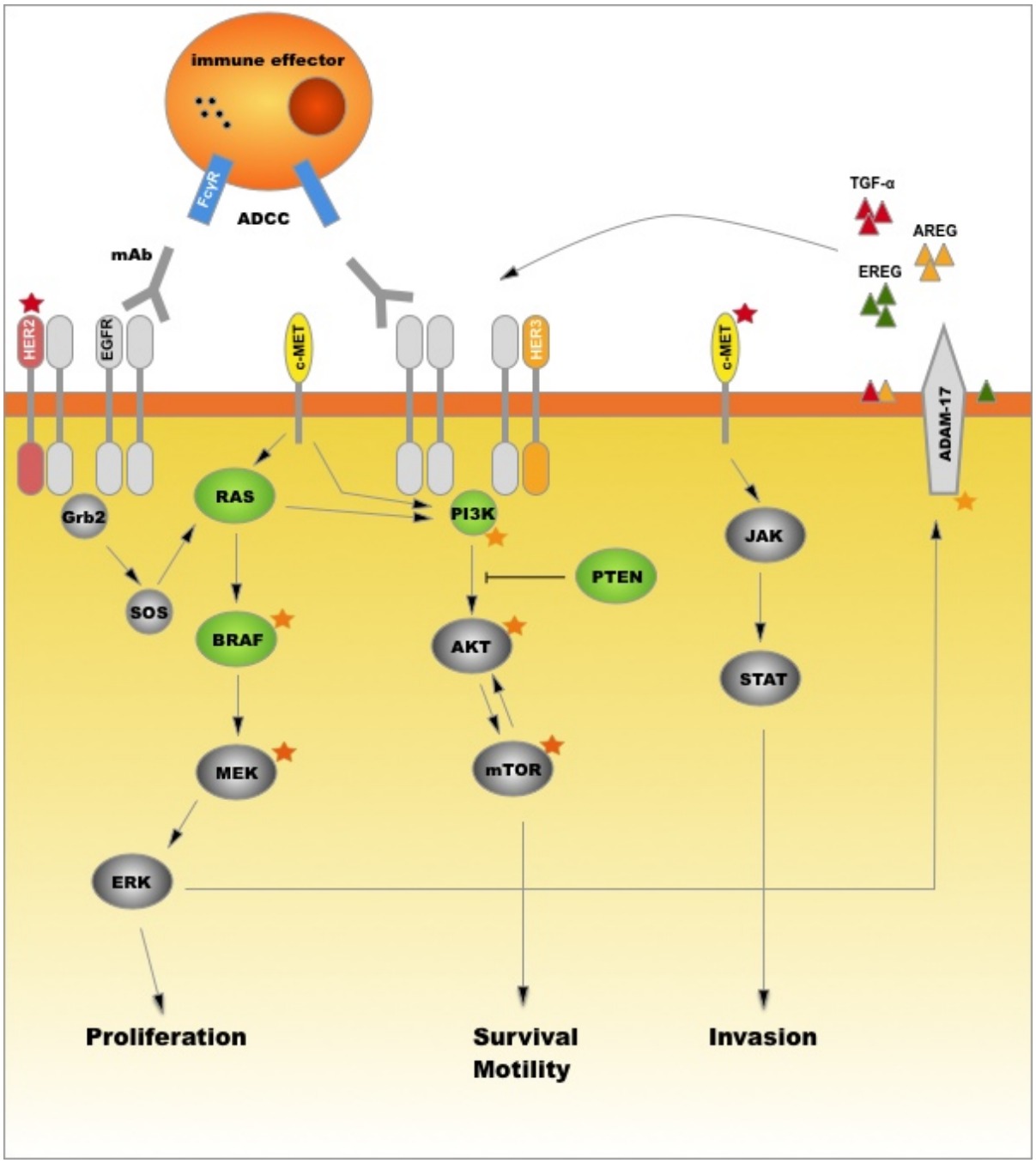

Figure 1: molecular determinants of resistance to EGFR-targeted therapy. Signaling pathways activated by EGFR homo- and hetero-dimers with tumor-promoting biological effects are depicted, along with signaling originating from alternative receptors such as c-MET; signaling is activated through autocrine and paracrine action of EGFR ligands secreted by ADAM-17. Components of the network that are frequently mutated in mCRC with prognostic or predictive value are highlighted in green; potentially druggable targets are indicated with a red star. Immune effector cells (NK cells, macrophages, monocytes) induce ADCC when monoclonal antibodies (mAb) such as Cetuximab are bound to EGFR.