

Predictive Biomarkers to Anti-EGFR Inhibitors Treatment in the Management of Metastatic Colorectal Cancer

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Abstract

Despite the epidermal growth factor receptor (EGFR) monoclonal antibodies (moAbs), cetuximab and panitumumab, have expanded the range of treatment options for metastatic colorectal cancer (mCRC), the prognosis of these patients remains poor. In fact, resistance mechanisms limit the effectiveness of current cancer therapies to treat mCRC. The identification of resistance mechanisms may highlight new biomarkers useful to predict the clinical outcome or the likely responsiveness to pharmacological treatment of those metastatic CRC patients who cannot benefit to anti-EGFR moAbs. Data derived from multiple clinical trials have clearly demonstrated that KRAS mutations can be considered specific negative biomarkers of response to anti-EGFR monoclonal antibodies. Other molecular aberrations in the downstream pathway of EGFR such as BRAF, NRAS, and PIK3CA mutations, and PTEN loss are useful for selecting patients with reduced chance to benefit from anti-EGFR moAbs. The recognition of panels of biomarkers may suggest new strategies to overcome resistance by rational drug design and combination treatment. In this review we discuss the most recent data on predictive and prognostic biomarkers within the EGFR pathway, the challenges this emerging field presents and the future role of these molecular markers in CRC treatment.

Keywords: Colorectal cancer; Biomarkers; Anti-epidermal growth factor receptor (EGFR) therapy; KRAS; BRAF

Introduction

Colorectal cancer is the third most common cause of cancer related death in western societies, accounting for approximately 10% of all cancer incidence and mortality [1]. Despite advances in chemotherapy, the prognosis for patients with mCRC remains poor and most patients with metastatic cancer will succumb to the disease within 2 years of diagnosis. To improve survival, new therapeutic approaches focusing on the molecular mechanisms that mediate tumor cell growth or survival signals have gained much attention. In particular, in the last 5 years, cetuximab and panitumumab, two moAbs targeting the EGFR, have proven to be effective in combination with chemotherapy or as single agents for treatment of mCRC [2,3]. Both these drugs bind to the extracellular domain of EGFR, leading to inhibition of its downstream signaling. EGFR is a transmembrane tyrosine kinase receptor that, on ligand binding, triggers two main signaling pathways: the RAS-RAF-MAPK axis, mainly involved in cell proliferation, and the PIK3-PTEN-AKT pathway, essentially involved in cell survival and motility-invasion [Figure 1] [4]. Unfortunately, cetuximab and panitumumab are efficient in only a small percentage of patients and it is therefore extremely important to identify specific factors that will lead to clearer definition of those patients who will benefit from anti-EGFR treatments. In this review we aim to provide an overview of potential biomarkers analyzed as predictive factors for efficacy to anti-EGFR therapy in mCRC and, at the same time, we will

emphasize the challenges and controversies that withhold the clinical introduction of these biomarkers.

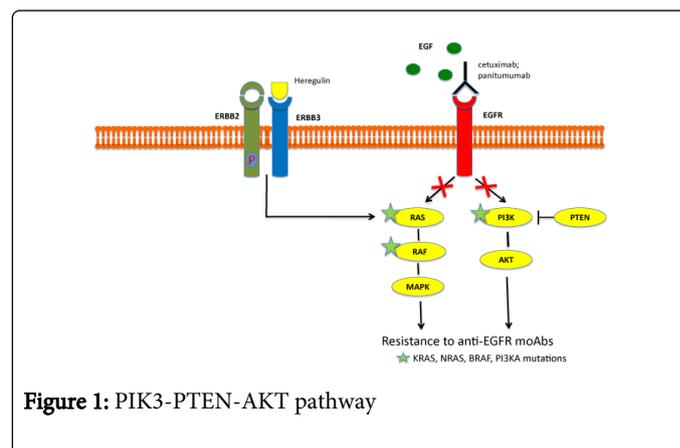


Figure 1: PIK3-PTEN-AKT pathway

EGFR

EGFR is a cell membrane growth factor receptor characterized by tyrosine kinase activity that plays a crucial role in the control of key cellular transduction pathways in both normal and cancerous cells. EGFR is overexpressed in a variety of human tumors, including colorectal cancer [5]. EGFR expression progressively increases with malignant transformation from normal colon, through adenoma, to the poorly differentiated and metastatic cancer, suggesting its role in oncogenesis. EGFR overexpression is observed in tumors with worse

histologic grade and lymph vascular invasion and it is associated with poor prognosis in the majority of studies [6]. In colorectal cancer an increase of EGFR protein expression, determined by immunohistochemistry (IHC), was initially selected as an entry criterion for early studies evaluating EGFR inhibitors on the assumption that sensitivity to such agents was associated with EGFR expression [7]. However, a large body of evidence from mCRC patients treated with moAbs [8-11] indicates that the degree of EGFR expression was not associated with clinical activity. In fact, several authors have reported that cetuximab was also active in tumors that were EGFR-negative by IHC [2,12]. Recently, retrospective analysis from the PRIME trial confirmed that, EGFR expression by immunohistochemical analysis was not predictive of efficacy to panitumumab combined with chemotherapy [13]. Many technical explanations have been advocated for the lack of association between EGFR protein detection by IHC and response to EGFR-targeted agents [14]. Based on this evidence, EGFR IHC expression is no longer considered a predictive factor for response to anti-EGFR treatments. Other potential CRC biomarker has been considered including the expression of EGFR in terms of gene copy number (GCN). Several studies have demonstrated that patients with increased EGFR GCN had better outcomes with anti-EGFR therapy compared with those whose tumors had normal EGFR GCN suggesting an association with treatment efficacy [15-19]. Moreover the value of EGFR status has been evaluated in patients with KRAS wild-type tumors who received cetuximab-based therapy in second-line treatment or beyond [20]. Response rates were significantly higher in patients with increased copy numbers of the *EGFR* gene [Table 1]. Although recent data are promising for the use of increased EGFR GCN as a positive predictive factor of clinical outcome to EGFR-targeted moAbs, the reproducibility of data remains the largest obstacle for clinical applicability of this molecular determinant. The available technologies for assessment of EGFR copy number, eg, fluorescence or chromogenic in situ hybridization (FISH or CISH), are easier to quantify compared with IHC, but the cutoff levels for significance are variable and copy numbers are often heterogeneous in metastatic disease, thus complicating clinical interpretation. Furthermore, significant inter-laboratory variability has been demonstrated in the measurement of EGFR copy number by FISH analysis [21]. Methods of tissue processing and EGFR scoring systems need to be standardized before using it for selecting patients for EGFR-targeted therapy.

Potential key factors in determining sensitivity to anti-EGFR therapies could be the overexpression of EGFR ligands, such as epiregulin (EREG) and amphiregulin (AREG). Elevated expression of epiregulin and/or amphiregulin may play an important role in tumor growth and survival by stimulating an autocrine loop through EGFR [22,23]. This may characterize a tumor that is EGFR dependent and, therefore, particularly sensitive to the ability of cetuximab and or panitumumab, to block ligand-receptor interaction. Several studies reported a strong correlation between increased tumoral mRNA of AREG, EREG and a clinical benefit from cetuximab (>50% reduction in the risk of death) [24-26]. Khambata-Ford et al. were the first to publish a gene signature obtained from snap-frozen liver metastasis of mCRC patients who were treated with cetuximab as monotherapy [27]. In this signature, two EGFR ligands, AREG and EREG, were found to predict cetuximab response. Tejpar et al. analyzing primary CRC formalin-fixed-paraffin embedded (FFPE) tumors from refractory metastatic patients treated with cetuximab-based therapy, confirmed the predictive value of EREG and AREG expression to

cetuximab sensitivity. In line with these findings Taberero et al. found that in mCRC patients receiving first line cetuximab combined with an irinotecan based regimen, AREG and EREG expression were elevated in tumors of patients without disease progression, either in the total population or in the KRAS wild type tumor subgroup [24]. Accordingly Taberero et al. showed that epiregulin and amphiregulin expression appeared to be elevated in tumors of patients with a favorable PFS [Table 1]. Conversely the opposite was found regarding TGF- α expression [28]. Gene expression of the two ligands, which are colocalised on chromosome 4q13.3, occurred more often in KRAS and BRAF wild type tumors. The correlation of low EREG/AREG expression with KRAS or BRAF mutated status could be due to the constitutive activation of the RAS/RAF/MAPK pathway which makes activation of the EGFR pathway redundant biologically. Alternatively, it could be due to a negative feedback loop linking MAPK axis activation with suppression of the EGFR pathway [29]. Evaluation of ligand expression by messenger RNA (mRNA) may be a useful prognostic marker in KRAS wild type (WT) patients regardless of receiving anti-EGFR therapy. In mCRC patients not treated with anti-EGFR moAbs, those with KRAS status and low EREG mRNA levels showed significantly better OS than those with high levels (p=0.006). AREG expression showed the same tendency but did not reach significant difference [30]. However, the available methodologies for quantification for ligand expression require validation with establishment of cutoff levels. The lack of standardized methodologies to quantify these markers has prevented AREG and EREG expression levels from being used as clinical biomarkers for directing treatment with EGFR-targeted agents.

Biomarker	Alteration frequency	and	Predictive and/or prognostic value	References
KRAS	Mutations: 30-40%	Codon 12: 70-80%	Predictive of resistance to anti-EGFR therapy	[13,33-42,47-51]
		Codon 13: 15-20%	Predictive of response to anti-EGFR therapy	[47-49]
NRAS	Mutations: 3-5%		Predictive of worse PFS	[45,47,54,55]
BRAF	Mutations: 10-15%		Both predictive and prognostic	[41,43,58,59]
EGFR	Copy number		Predictive of better prognosis	[15-21,24-27]
PIK3CA/PTEN	Mutations: 10-30%		Predictive of lack of response	[25,63-73]
HER 3	Overexpression		Resistance to cetuximab	[75]
HER 2	Amplification: 2%		Resistance to cetuximab	[76-80]
MET	Amplification and phosphorylation		Resistance to cetuximab	[81-84]

Table 1: Clinical biomarkers of resistance to EGFR-targeted therapies in CRC.

KRAS

Kirsten (K)RAS, a member of the rat sarcoma virus (ras) gene family of oncogenes (including KRAS, HRAS and NRAS) encoding

guanosine di/triphosphate-binding proteins, act as an important effector of EGFR. It is a proto-oncogene encoding a small 21 kD guanosine diphosphate (GDP)/guanosine triphosphate (GTP) binding protein RAS that acts as a self-inactivating intracellular signal transducer [31,32]. Activating KRAS mutations result in a constitutively active GTP-bound protein which constitutively leading the cells to become independent from the EGFR signaling activation [Figure 1]. Somatic mutations of KRAS occur in 30-40% of CRC and mostly occur in codon 12 (about 70-80%) and codon 13 (about 15-20%) of exon 2 [Table 1]. The remaining mutations are mainly located on codons 61, 146 and 154 [33]. Initial retrospective analyses revealed that patients with CRC carrying activating KRAS gene mutations do not benefit from cetuximab therapy [35]. The discrete number of mutations, as well as the inherent stability and the detection of KRAS mutations have made it the major predictor for resistance to anti-EGFR moAbs [Figure 1]. This fact has been consistently shown in small single-arm data sets [15,33,35-37] but also in retrospective analysis of large phase III studies and some prospective trials of patients receiving first [13,38-41] and subsequent lines of treatment. In these studies, patients with metastatic CRC harboring KRAS mutations did not extract any benefit of treatment with cetuximab or panitumumab either alone or in combination with standard CT. This discovery led to the first practical implementation of personalized medicine in metastatic CRC. The evidence that KRAS mutations were associated with the lack of response to cetuximab in chemorefractory mCRC patients, leading the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to restrict the use of cetuximab monotherapy or in combination with chemotherapy, only to patients with KRAS WT tumors [42]. The CRYSTAL and OPUS studies confirmed the consistency of the benefit obtained from adding cetuximab to first-line chemotherapy in patients with KRAS WT metastatic CRC: 845 KRAS exon 2 WT patients treated with cetuximab plus chemotherapy were evaluated and these patients had a significant in OS (hazard ratio [HR] 0.81; $p=0.0062$), PFS (HR 0.66; $p<0.001$) and overall response rate (ORR odds ratio 2.16; $p<0.0001$) [41]. The Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy (PRIME) was the first phase III trial to evaluate the addition of panitumumab to FOLFOX4 for the initial treatment of patients with KRAS WT metastatic CRC [13]. Douillard et al. demonstrated an improvement in terms of OS and PFS for patients treated with addition of the anti-EGFR monoclonal antibody only patients with KRAS WT tumors (PFS: 9.6 vs. 8 months; HR 0.80; 95% CI, 0.66 to 0.97; $p=0.002$; OS: 23.0 vs. 19.7 months; HR: 0.83; 95% CI, 0.66 to 0.97; $p=0.02$), whereas in the mutant RAS setting PFS and OS were significantly reduced [43]. Although these trials confirmed the negative predictive value of KRAS mutation for benefit with anti-EGFR therapy and a recent meta-analysis is in line with them [44, Table 1], the results have not been confirmed by other academic studies. COIN trial did not show benefit with the addition of cetuximab to oxaliplatin-based CT in KRAS WT population [45], as well as NORDIC VII trial showed that cetuximab in combination with the continuous or intermittent FLOX regimen did not improve efficacy compared to FLOX alone, with no evidence of KRAS status for a predictive value in terms of cetuximab efficacy [46]. Some concerns have been raised about the consecution of this trial, including the choice for different oxaliplatin-based schedules (mFOLFOX6 or XELOX [capecitabine/oxaliplatin]) as the chemotherapy backbone. Subgroup analysis suggests that patients receiving oxaliplatin and infusional 5-FU could derive some benefit from the addition of cetuximab. In the recently presented NORDIC trial of FLOX (bolus 5

FU/folinic acid and oxaliplatin) with or without cetuximab, KRAS mutation status was not predictive for anti-EGFR moAbs efficacy [46]. Interestingly, retrospective studies have shown that some patients with KRAS mutations respond to cetuximab or panitumumab [47,48]. In fact, recently it has been reported by De Roock *et al.* that chemorefractory mCRC patients which carry a KRAS mutation in codon 13 may have longer PFS (median, 4.0 vs. 1.9 months) and longer OS (median, 7.6 vs. 5.7 months) compared with patients harboring other KRAS mutations when treated with cetuximab [47]. Tejpar et al. showed a similar benefit, although only for PFS.56 However these were small studies, with only 32 and 83 patients, respectively, with the G13D mutation. On the other hand, a number of studies have demonstrated no benefit for either cetuximab or panitumumab by specific KRAS mutations [49]. The evidence of some KRAS G13D not achieving a clinical benefit from anti-EGFR-targeted treatment indicates that these results have to be considered with caution and their impact should be carefully assessed in well-designed prospective trials. However additional prospective studies are needed to confirm this finding and to validate G13D and other mutations in KRAS as positive biomarkers of response to EGFR-targeted therapies [Table 1]. Additional RAS mutations predicted a lack of response in patients who received the anti-EGFR treatment. The recent data underline the importance of a complete RAS mutational analysis (i.e.: KRAS and NRAS analysis of exon 2 [codons 12/13], exon 3 [codon 59/61] and exon 4 [codon 117/146]) to determine the real benefit of a first-line anti-EGFR treatment in terms of ORR, PFS and OS in RAS WT patients than RAS mutated ones [44,51,52]. On the basis of these initial data, On 21 November 2013, the Committee for Medicinal Products for Human Use (CHMP) of European Medicines Agency (EMA) changed the indication for use of cetuximab in metastatic colorectal cancer, the patients who will undergo to treatment are not only be defined as "KRAS wild-type patients", but, in a more comprehensive way, as "RAS wild-type patients".

N-RAS

NRAS gene codes for a protein, N-Ras that is an alternate effector of K-Ras. Recent pre-clinical data show that *NRAS* strongly promotes tumorigenesis in an "inflammation context". Moreover, *NRAS* mutations determine the pro-tumorigenic nature of this gene not so much promoting proliferation and suppressing differentiation, but rather inducing the overexpression of its anti-apoptotic function, which is mediated by the activation of a non-canonical MAPK pathway that signals through Stat3. These findings led to the hypothesis that the small subset of NRAS mutated colorectal cancers may arise in tumors that develop in a background of constant apoptotic stimulus, for example, in cancers that arise in individuals with chronic inflammation of the gastrointestinal tract [31,52,53]. Mutations within this gene are found in 3-5% of mCRC patients [Table 1]. Although a retrospective study and the PICCOLO (Panitumumab, Irinotecan and Ciclosporin in Colorectal Cancer Therapy) trial have demonstrated a reduced response to cetuximab and panitumumab for patients with NRAS mutations [47,54] further work is required to demonstrate the predictive capacity of these mutations. In the COIN trial, NRAS mutated patients (NRAS mutation rate: 4%) had worse PFS than those harbouring NRAS WT tumors, independently from the treatment received ($p<0.0088$) [45, Table 1]. Lambrechts et al. analyzed NRAS codon 12 and 13 mutation status in patients with chemorefractory mCRC treated with cetuximab with or without irinotecan [55]. Five of 98 (5%) patients with KRAS wild-type tumors had NRAS mutation, and none showed an objective

response. On the basis of these results a prevalent role of NRAS as prognostic factor can be identified, even though it should be noted that the relatively small number of patients harboring the mutation and the potential presence of confounding factors (such as a difference in number of chemotherapy lines received, in particular for patients with poor performance status) cannot preclude a potential role as predictive factor also. Based on these data, NRAS mutational analysis cannot represent a reliable asset in the clinical practice.

BRAF

The BRAF gene encodes a protein kinase that is the direct and immediate downstream effector of KRAS in the RAS/RAF/MAPK pathway [Figure 1]. V600E is the most common oncogenic mutation of BRAF, accounting for nearly 90% of all mutations, and has been identified in 10% to 15% of colorectal cancers [56,57, Table 1]. Several studies show that, in the CRC chemorefractory setting, BRAF mutations are predictive of resistance to EGFR-targeted moAbs. Conversely, in first-line setting the predictive role of BRAF mutational status to anti-EGFR therapy has not been clearly demonstrated. Laurent-Puig et al. and Park et al. in their analyses suggested that patients with KRAS wild-type tumors, BRAF mutations were weakly associated with lack of response ($P=0.063$) but were strongly associated with shorter progression-free survival ($P<0.001$) and shorter overall survival (OS; $P<0.001$) [58,59]. In the CRYSTAL and OPUS study, BRAF mutations were indicated as prognostic rather than predictive markers [41, Table 1], both these study showed a decreased PFS and OS in the BRAF mutant population, regardless of treatment arm. OS for the KRAS/BRAF WT group treated with the combination arm was 25.1 vs. 21.6 months of the control arm. In the KRAS WT/BRAF mutant group, OS was 14.1 vs. 10.3 months respectively. The recent analysis performed by Douillard about efficacy of an anti-EGFR treatment plus chemotherapy according to RAS and BRAF status also suggested that BRAF V600E mutations confer a poor prognosis in terms of median OS, regardless of the treatment group [42]. Globally BRAF mutations seem to represent a possible prognostic marker rather than predicting efficacy of anti-EGFR therapies, particularly in first-line setting. The selective BRAF inhibitor, PLX4032, vemurafenib, effective in the treatment of melanoma, demonstrated a modest benefit in colon cancer patients harboring the same BRAF (V600E). Prahallad et al. recently tried to investigate the cause of the limited therapeutic effect of PLX4032 in BRAF(V600E) mutant colon tumors. They find that inhibition of EGFR by the antibody drug cetuximab or the small-molecule drugs gefitinib or erlotinib is strongly synergistic with BRAF (V600E) inhibition, both in vitro and in vivo, through feedback activation of the EGFR-downstreaming, which supports continued proliferation in the presence of BRAF(V600E) inhibition. Melanoma cells express low levels of EGFR and are therefore not subject to this feedback activation. These data suggest that BRAF (V600E) mutant colon cancers, for which there are currently no targeted treatment options available, might benefit from combination therapy consisting of BRAF and EGFR inhibitors [60,61].

PIK3CA/PTEN

PIK3s belong to a family of intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides. The lipid products of PIK3 reactions create binding sites for specific, lipid-binding domains on many intracellular signaling proteins and there by regulate a range of cellular processes,

including cancer-cell proliferation, survival, motility, and metabolism. The somatic mutations in cancers are present in the PIK3R1 gene (encoding for p85 α , the regulatory subunit) and in the PIK3CA gene (encoding for the p110 α , the catalytic subunit). In colorectal cancer, PIK3CA mutations are concentrated in 2 hot spots of the gene: 60-65% of mutations are single amino acid substitutions located in codons 542 and 545 of exon 9 (coding for the helical domain), while 20-25% are located in codon 1047 of exon 20 (coding for the kinase domain of the protein), thereby conferring a gain of transformative enzymatic function [62-64]. In the absence of PIK3CA mutations are classically activated by receptor tyrosine kinases or activated RAS and thereby recruited to the plasma membrane to convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) [24-26]. PIP3 provides docking sites for signaling proteins such as PDK1 (3'-phosphoinositide-dependent kinase-1) and serine-threonine kinase AKT (also known as protein kinase B or PKB). In contrast, balancing PIK3CA pathway activation, the tumor suppressor PTEN (phosphatase and tensin homolog) antagonizes PIK3CA activity by dephosphorylating PIP3. Preclinical data have demonstrated the importance of a functioning PTEN/PIK3/AKT pathway in determining the sensitivity of CRC cell lines to cetuximab [Figure 1]. In literature, PIK3CA mutations have been described in 10-30% of unselected CRC patients [65, Table 1], and sometimes can occur in conjunction with KRAS, NRAS, and BRAF mutations [48]. Activating mutations of PIK3CA and PTEN loss confer resistance to cetuximab-induced apoptosis [66]. De Roock et al. in a large retrospective study showed that tumors with PIK3CA activating mutations in the exons 20 were associated with worse outcomes after cetuximab therapy compared with PIK3CA wild-type. Mutations in exon 9 had no significant effect on RR, PFS, and OS. These data suggests the role of PIK3CA exon 20 mutations as potential biomarker for resistance to anti-EGFR moAbs but only in KRAS wild-type mCRC. In fact the predictive power of PIK3CA is expected to be the highest in KRAS wild-type patients, intermediate in those with unknown KRAS status, and lowest in KRAS mutant patients [67]. Saridaki et al. in their study demonstrated that there was no significant correlation between the time to tumor progression (TTP) and OS and the PIK3CA mutational status when the analysis was performed in the whole group of patients; however, when only KRAS wild-type patients were analyzed, PIK3CA mutational status was correlated with a significantly lower TTP [25]. In conclusion, PIK3CA exon 20 mutations may be a potential biomarker for resistance to anti-EGFR therapy in KRAS WT patients [Table 1], but these data, due to the low frequency of these mutations, should be regarded as hypothesis-generating and nowadays require confirmation in large randomized clinical trials according to PIK3CA exon mutational status, in particular in KRAS WT and anti-EGFR untreated settings. It has been also demonstrated that PIK3CA mutation is a poor prognostic factor for rectal cancer [68]. A study showed that 7.9% of 240 resectable stage I/III rectal tumors harbored PIK3CA mutations, most of them on exon 9. Patients with PIK3CA mutations experienced more local recurrences compared with patients without mutations, as well as earlier recurrences after surgery (median local recurrence-free interval from surgery of 7.9 vs. 19.6 months; $P=0.07$).

The role of PTEN loss and consecutive over-activation of the AKT pathway and its evaluation is still under investigation, as far as response to anti-EGFR moAbs is concerned. Several studies shown that PTEN status is associated with objective responses in cetuximab-treated mCRC patients suggesting that PTEN-positive tumors tend to

have a better outcome than negative ones [60-72]. This evidence is in contrast with the study of Sartore-Bianchi, where the authors showed that patients with tumors harboring PTEN had a worse clinical outcome in terms of PFS, compared with wild-type tumors that reached statistical significance if there was the combination with the PIK3CA mutation [73]. This contrast data probably could be due to several methodological differences such as the used anti-PTEN antibodies, the IHC scoring algorithms and cut-off criteria. In conclusion, since PTEN IHC is not yet adequately validated, it cannot be considered for immediate routine clinical use, but it should be kept in mind in the planning process of prospective biomarkers studies.

Other Potential Biomarkers

HER-3

The efficacy of anti-EGFR moAbs in the medical management of mCRC patients is limited by de novo and acquired resistance mechanisms. The molecular bases of secondary resistance to cetuximab in CRC are poorly understood. KRAS mutations, but also NRAS, BRAF and PIK3CA/PTEN alterations have been mainly assessed as markers of resistance to anti-EGFR therapy [74]. Several other hypotheses have been proposed, in this scenario Scartozzi et al. [75] evaluated the role of HER-3 as indicator of worse outcome in metastatic colorectal cancer patients treated with Cetuximab, and found a statistically significant relationship between HER-3 IHC overexpression and resistance to Cetuximab respectively in patients with or without HER-3 overexpression, RR 18% vs. 42%, PFS 2.8 vs. 6.3 months, OS 10.5 vs. 13.6 months [Table 1].

HER-2

Nowadays, literature data suggest the cumulative evidence that HER-2 is involved in CRC cell growth and progression and that HER-2, which can be considered the major EGFR partner, could modify the sensitivity to anti-EGFR treatments [Figure 1]. In 2011, two pivotal papers have been published almost simultaneously about this aspect. Yonesaka et al. showed that cetuximab-resistant cancer cells (both in culture and in patients) are able to up-regulate signaling by HER-2 gene amplification or by up-regulation of the HER-2/HER-3 ligand heregulin. Notably, the authors suggested that HER-2 amplification and heregulin overexpression are peculiar and unique mechanisms of anti-EGFR treatment resistance, also because they represent a clear example of de novo or acquired resistance mechanisms that lead to activation of a bypass signaling pathway, which is biologically feasible because HER-2 is not a direct or indirect target of cetuximab treatment. Moreover, Yonesaka reported that inhibition of HER-2 or disruption of HER-2/HER-3 heterodimerization restored cetuximab sensitivity both in vitro and in vivo, suggesting that HER-2 inhibitors, in combination with cetuximab, may represent a rational therapeutic strategy that should be assessed in patients with cetuximab-resistant cancers [76]. Bertotti et al. created a large xenograft cohorts from 85 patient-derived, genetically characterized metastatic colorectal cancer samples (called 'xenopatiens'), and this approach led to the identification of HER-2 amplification as an additional molecular biomarker of resistance to EGFR-targeted therapy with cetuximab in xenopatiens affected by tumors for which other known molecular alterations conferring resistance were excluded, i.e., KRAS, NRAS, BRAF and PIK3CA mutations. In terms of "personalized medicine", the authors reported two main novel findings. First, they observed a greater frequency of

HER-2 amplification in cetuximab-resistant cases and its progressive enrichment along with refinement of genetic selection, showing that HER-2 amplification, which occurred in a small percentage (2-3%) of genetically unselected CRCs [Table 1], was detected in six out of 44 cases (13.6%) in KRAS WT CRC patients that displayed de novo resistance to anti-EGFR treatment, and even reached a value of 36% (4/11 cases) in KRAS/NRAS/BRAF/PIK3CA WT xenopatiens in which cetuximab treatment was ineffective. Second, Bertotti et al. reported that dual targeting of HER-2 and EGFR induced overt and long-lasting tumor regression when combinations of lapatinib (a small dual EGFR/HER-2 inhibitor) and pertuzumab (a HER-dimerization inhibitor humanized monoclonal antibody) or, to a lesser extent, lapatinib and cetuximab were used [77]. To confirm the role of HER-2 as a novel CRC biomarker and as an essential driver of tumor growth, recently Martin et al. evaluated the HER-2 gene status by FISH in 170 KRAS WT metastatic CRC patients treated with cetuximab or panitumumab and, according to HER-2 gene copy number status, authors found that patients were characterized by three distinct cytogenetic patterns: 4% of cases had HER-2 gene amplification in all neoplastic cells (this subgroup was called by authors "HER-2-all-A"), 61% of patients had HER-2 gain due to polysomy or to gene amplification in minor clones (called "HER-2 FISH +"), while 35% of patients had no or slight HER-2 gain (and so called "HER-2 FISH-"). The results showed, in agreement with results of Bertotti and Yonesaka, that in KRAS WT patients the "HER-2-all-A" status conferred resistance to cetuximab or panitumumab and worst outcome, while patients with "HER-2 FISH-" profile had an intermediate behavior, and patients with "HER-2 FISH +" profile were related to the highest survival probability (median PFS in months: 2.5 vs. 3.9 vs. 7.6, respectively; median OS in months: 4.2 vs. 9.7 vs. 13, respectively; PFS: $p < 0.0001$; OS: $p < 0.0001$). To explain the partially surprising survival results seen in the "HER-2 FISH +" population, authors postulated that in these cases HER-2 deregulation could derive from chromosome instability, with HER-2 polysomy due to a general polyploid karyotype, and that response to anti-EGFR treatment could be related not so much to HER-2 gene itself but rather to the EGFR gene or to a complex karyotype [78]. However, studies or clinical trials using anti-HER-2 therapy in CRC are rare. To our knowledge, in the only published clinical report, Ramanathan et al. performed a phase II trial where a combination therapy with trastuzumab and irinotecan was evaluated in 138 pre-treated CRC patients: the authors found only 8 HER-2 amplified patients, and this low accrual led to the premature closure of the study, but partial responses were described in 5 of 7 evaluable patients; even if the sample was very small, these results are significant if we consider that authors defined as partial response a >50% decrease in the sum of the products of the measured lesions (differently from RECIST 1.1 criteria where the cut-off is a >30% decrease) and, notably, that responses were maintained for at least 6 weeks in 4 patients [79]. Actually, the combination of trastuzumab with either lapatinib or pertuzumab is being investigated in a pioneer phase II multi-center 2-sequential cohorts trial: this study, that is actively recruiting also in our center, is designed for KRAS WT metastatic CRC patients harboring HER-2 SISH amplification, after failure of previous fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab, cetuximab or panitumumab containing regimens, and has the primary endpoint of objective response rate [80]. In conclusion, all these findings indicate that HER-2 really could be in the next future a novel biomarker virtually very useful for a "super selected" setting of metastatic CRC patients, because the amplification of these oncogene is associated not only with resistance to anti-EGFR treatment [Table 1], but may also identify patients who could benefit

from specific anti-HER-2 drugs or from combined EGFR/HER-2 dual blockage.

MET

MET is a RTK involved in cellular proliferation and apoptosis. Activation of MET may lead to the activation of pathways downstream of RAS, such as Raf/MEK/MAPK and the PIK3/protein kinase B pathway (PKB). In addition, MET is able to directly activate PIK3/PKB pathway in a RAS independent manner [81]. HGF/MET axis seems to play an emerging role to predict response to anti-EGFR treatment in advanced CRC, as this pathway may represent a parallel signalling by which colorectal cancer cells are able to achieve an anti-EGFR resistance. Nowadays, the data remain mostly in the preclinical phase and clinical results regarding MET expression and different outcome for mCRC patients treated with anti-EGFR inhibitors, are rather scarce. In preclinical model, we have demonstrated that MET activation may play a relevant role in determining acquired resistance to cetuximab in CRC cell lines [82, Table 1]. In fact, activated MET was detected only in CRC cells with acquired resistance to cetuximab and inhibition of MET expression by siRNA restored cetuximab sensitivity. Moreover the treatment of cetuximab with PHA665752, a selective MET inhibitor, restored the sensitivity to cetuximab in a dose-dependent manner suggesting that the combined inhibition of EGFR and MET receptor could represent a strategy for preventing and/or overcoming cetuximab resistance in CRC patients. Interestingly, overexpression of transforming growth factor α (TGF α) induced the EGFR-MET interaction, with subsequent MET phosphorylation and activation of MET downstream effectors in cetuximab-resistant CRC cells [82]. In line with these findings Bardelli et al. have demonstrated the predictive role of MET both in primary and in acquired resistance to anti-EGFR treatment [Table 1]. About primary resistance, the authors reported that “quadruple negative” CRC cell lines (i.e.: KRAS, BRAF, NRAS, and PIK3CA genes wild-type cells) developed cetuximab and panitumumab resistance when ectopic MET overexpression was induced by cDNA transfection. Moreover, experiments on xenografts models derived from patients anti-EGFR “naive” showed that cetuximab treatment was ineffective in mice engrafted with CRC specimens carrying MET amplification, thus suggesting that MET amplification may be involved in primary resistance to anti-EGFR monoclonal antibodies. About secondary resistance, the authors carried out a molecular analysis on tissue samples obtained before anti-EGFR treatment and at the time of CRC progression, and found that in patients with no known mutation related to anti-EGFR therapy (i.e. KRAS, BRAF, NRAS, PIK3CA wild type and HER-2 not amplified patients), MET amplification and overexpression was the only mechanism of acquired resistance observed, with the exception of one case, in which rare MET-amplified cells were found in tissue sample before treatment. Moreover, the prevalence of MET amplification observed by Bardelli in untreated metastatic CRC setting was around 1% (2 of 196 samples), while this percentage increases to 12.5% when were considered only “quintuple-negative” samples not responsive to cetuximab emphasizing the concept of “the best molecular profile for the best tailored treatment” [83]. The potential prognostic role of MET in cetuximab treated patients has been investigated by Inno and colleagues. They performed an analysis of 73 metastatic CRC patients treated with cetuximab-containing regimens, and reported that, compared with low/normal expression, MET overexpression significantly correlated with shorter median PFS and median OS [84].

Conclusions

The introduction of moAbs that target EGFR not only have expanded treatment options for mCRC but also have defined new paradigms in medical oncology in the field of personalized therapy. Although the discovery of RAS mutations has paved the way to select the patient to treat with this drugs, the onset of novel biomarkers for assessment of anti-EGFR based therapy resistance is creating a problem in understanding where to focus our attention. One critical aspect is the definition of an ideal biomarker. An ideal biomarker may be suitable for the early diagnosis of a disease; a biomarker may also appear or disappear over the course of disease progression and thus be useful in determining the prognosis of a disease within an individual. The ideal biomarker should be easily obtained with minimum discomfort or risk to the patient. In addition, a reliable biomarker will have a detection method that is both sensitive and specific and is highly reproducible among clinical laboratories. Unfortunately this hypothesis is not applicable to clinical practice, the methodologies for these and many of the biomarkers described here require further standardization and validation. Both these findings and the complexity and heterogeneity of molecular alterations that are being identified as resistance mechanisms to anti-EGFR therapies suggest that a comprehensive molecular characterization of mCRC will likely be necessary in the next future in order to choose the most appropriate therapy for each individual patient. Finally, it is possible that in the near future patients with mCRC will have a complete molecular profile analysis of their tumors so that targeted therapies will be administered effectively on specific subsets of patients with genetic aberrations.

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