Strategies to overcome resistance to anti-EGFR monoclonal antibodies in colorectal cancer

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ABSTRACT
In the past few years, knowledge on the resistance to therapy with monoclonal antibodies in colorectal cancer has evidenced the role of mutations in a series of proteins involved in the epidermal growth factor receptor (EGFR) pathway. KRAS mutations can influence the response to therapy, as demonstrated by clinical utilization of cetuximab and panitumumab that are indicated only in KRAS wild-type colorectal cancer. Recently, it has been demonstrated that the mutational status of other proteins like NRAS, BRAF, PI3K or alterations in EGFR and other tyrosine kinase receptors are involved both in primary and secondary resistance to anti-EGFR monoclonal antibodies and could predict the patient response and survival. Many therapeutic strategies have been studied to overcome resistance to these drugs, which include the use of drugs directed against proteins involved in the intracellular signalling pathway of EGFR, such as inhibitors of MEK, BRAF, PI3K/mTOR, or directed against other tyrosine kinase receptors such as MET and HER2. This review will analyse recent strategies utilized to overcome resistance to anti-EGFR monoclonal antibodies in colorectal cancer.

KEYWORDS: colorectal cancer, cetuximab, mAbs resistance, panitumumab

ABBREVIATIONS
CRC : colorectal cancer
EGFR : epidermal growth factor receptor
EMA : European Medicines Agency
ERK : extracellular signal-regulated kinases
FOLFIRI : folinic acid, fluorouracil, irinotecan
FOLFOX : folinic acid, fluorouracil, oxaliplatin
HER2 : human epidermal growth factor receptor 2
KRAS : V-KI-RAS2 kirsten rat sarcoma viral oncogene homolog
mAb : monoclonal antibody
MAPK : mitogen-activated protein kinase
mCRC : metastatic colorectal cancer
OS : overall survival
PFS : progression-free survival
PI3K : phosphatidylinositol-4,5-bisphosphate 3-kinase
PR : partial response
PD : progression disease
PTEN : phosphatase and tensin homolog
RAF : rapidly accelerated fibrosarcoma
RR : response rate
SD : stable disease
VEGFR2 : vascular endothelial growth factor 2

INTRODUCTION
Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world: it is estimated that there are more than 1.4 million men and women in the United States with a previous CRC diagnosis, and an additional 134,490 cases were diagnosed in 2016 [1, 2]. CRC is the third cause of cancer deaths among men and women in the United States [2] and the second in European Union [3]. The prognosis of patients with metastatic CRC (mCRC) has improved in the last 15 years,
thanks to the introduction of chemotherapy drugs such as fluoropyrimidines, oxaliplatin, and irinotecan, and molecular targeted drugs such as regorafenib, aflibercept and monoclonal antibodies (mAbs) like cetuximab, panitumumab and bevacizumab [4, 5].

Cetuximab and panitumumab are mAbs directed towards epidermal growth factor receptor (EGFR); they bind to the extracellular domain of this receptor, thus inhibiting its activation and its downstream intracellular signals, in particular the RAS–RAF–MEK–MAPK and the PTEN–PIK3CA–AKT pathways [6]. Undoubtedly they represent the greatest step towards the treatment of CRC in terms of progression-free survival (PFS), overall survival (OS), response rate (RR), and quality of life among different lines of treatment [7]. Unfortunately, anti-EGFR antibodies are effective only in a restricted subset of patients because of the development of several mechanisms of resistance and the lack of clinical and molecular biomarkers that could predict treatment response. When cetuximab and panitumumab were used as single agents in unselected patients with chemotherapy-refractory mCRC, they achieved a RR of only 10% [8, 9]; CRC often harbour genetic alterations in proteins of the EGFR pathway responsible for the treatment failure (intrinsic or primary resistance). Moreover, even in patients who initially respond to anti-EGFR monoclonal antibodies, the duration of this response is restricted and does not last more than 3-18 months, because of the appearance of genetic alterations responsible for acquired or secondary resistance [10].

Genomic alterations in the downstream effectors of the EGFR pathway are the most common mechanisms of resistance; first of all, randomized phase III trials demonstrated that chemorefractory mCRCs carrying KRAS mutations were resistant to the treatment with anti-EGFR mAbs, and therefore both Food and Drug Administration and European Medicines Agency (EMA) restricted the use of cetuximab and panitumumab to patients with KRAS wild-type mCRC [4, 11, 12].

Recently, other alterations in EGFR signalling pathway are recognized as involved in anti-EGFR drug resistance, such as mutations in NRAS and BRAF [13], alteration in PI3K/AKT/mTOR pathway [13-15], amplification of HER2 [16], and mutations in tyrosine kinase receptors such as EGFR [17, 18], HER3 [19] and MET [20].

These genetic alterations are biochemically responsible for the activation of MEK/ERK pathway and have been recognised in both primary and secondary resistance, except for rare mutations in the extracellular domain or in tyrosine kinase domain of EGFR, which have been only identified for acquired resistance [10].

**RAS/RAF**

RAS mutations have a key role in the mechanisms of both primary and acquired resistance to anti-EGFR mAbs. RAS mutations compromise the intrinsic ATPase activity of RAS, causing an accumulation of mutant proteins in the active conformation, thus leading to constitutive activation of MAPK pathway even in the presence of EGFR inhibitors [7, 21]. The RAS genes have been reported to be mutated in approximately 40%-50% of CRCs [22]; the most common RAS mutation is in KRAS exon 2 (42.6%), followed by KRAS exon 3 (3.8%) and KRAS exon 4 (6.2%), NRAS exon 2 (2.9%), NRAS exon 3 (4.2%) and NRAS exon 4 (0.3%) [21, 23]. The most frequent KRAS exon 2 mutations are in codons G12D, G12V and G12C, KRAS exon 3 are in codons Q61H and Q61R, KRAS exon 4 are in codons A146T and A146V, NRAS exon 2 in codon G12D, NRAS exon 3 in codons Q61K and Q61R and NRAS exon 4 in codon A146T [21].

Preclinical data and retrospective analysis from phase III clinical studies suggested that not all KRAS mutations have the same negative predictive role and that patients with KRAS G13D mutation can achieve a benefit from cetuximab in both first-line and advance-line treatments [24, 25]. This observation was not confirmed by meta-analysis and prospective trial, in which no statistical difference in terms of PFS or OS was observed between patients carrying KRAS G13D and other KRAS mutations with cetuximab monotherapy or cetuximab plus irinotecan [26, 27].

The mutational status of KRAS is concordant between primary tumours and metastasis, suggesting that these mutations have an essential role in the
early phase of tumorigenesis and that they are conserved during tumour progression [7].

These mutations also drive secondary resistance to anti-EGFR therapy in 50-80% of patients; they may be present in a small fraction of cells within the tumour before treatment initiation and then may be selected by pressure from the anti-EGFR treatments or arise as a result of continued mutagenesis during the treatment [28, 29]. In both primary and secondary resistance, a small percentage of patients (0.7%) present KRAS amplification [30] that seems to be mutually exclusive with other KRAS or BRAF mutations.

10-20% of patients with CRC carried a mutation on BRAF, a serine/threonine protein kinase which is the first downstream effector of RAS [31, 32]; BRAF mutations have been also reported in melanoma, thyroid and ovarian cancer with a frequency of up to 70%, 45%, and 30%, respectively [31, 33, 34].

The most common BRAF mutation is the V600E point mutation in the kinase domain, derived from a thymidine-to-adenine substitution at nucleotide 1799, which results in the replacement of valine 600 mostly with glutamic acid. This point mutation alters the conformation of the catalytic domain activating the protein kinase activity and the downstream ERK/MAPK pathway [21, 31, 35]. This mutation accounts for 80% of all BRAF mutations [31]. Several clinical studies have demonstrated that the presence of BRAF mutations predicts resistance to anti-EGFR therapies and is a marker of poor prognosis. Even though BRAF V600E is generally mutually exclusive with RAS mutations, recent studies have demonstrated the coexistence of BRAF mutations with others, including TP53, KRAS and PIK3CA exon 9 and exon 20 [7, 36, 37]. While the association of the BRAF V600E mutations and colon cancer mortality is well established [38, 39], its role as a predictive biomarker to anti-EGFR treatments is not clearly understood: several studies have showed that patients carrying a BRAF mutation do not achieve any benefit from anti-EGFR treatments in second-line or in later lines of therapy [38, 40]. However, data from the first-line setting are often contradictory [20, 41-43]. Probably, the small percentage of BRAF mutated patients, together with a lack of prospective studies, do not allow establishing the predictive role of BRAF mutation for treatment with cetuximab and panitumumab. BRAF mutation has been also recognized as a mechanism of acquired resistance to anti-EGFR therapy [44].

**PI3K/PTEN**

Alterations in genes that encode for PIK3CA/AKT/mTOR signalling pathway are involved in the development of malignant tumours and could impair the response to anti-EGFR mAbs. Mutations of PIK3CA and loss of PTEN, which result in the pathological activation of this pathway, were observed in patients with CRC resistant to anti-EGFR antibodies: they occur in 10-20% and 30% of CRCs, respectively [45]. Even though in preclinical models the alterations in this pathway were found to be predictive of the therapy failure with these drugs [46, 47], they were not clinically validated as predictive markers because PI3KCA and PTEN alterations are often concomitant with RAS and/or BRAF mutations, and PTEN expression has been associated with outcome only in metastases but not in primary tumours [48]. Moreover, only PIK3CA exon 20 mutations were predictive of a lack of response to cetuximab in the KRAS wild-type subpopulation, whereas PIK3CA exon 9 mutation was associated with KRAS mutations, suggesting a secondary role of PIK3CA exon 9 mutations in cetuximab efficacy [49, 50].

**Alteration in tyrosine kinase receptors**

Both HER2 amplification and HER3 mutations were associated with poor response to anti-EGFR antibodies. HER2 amplification has been described in a small percentage of CRC patients (2-3%), but the frequency was higher in KRAS wild-type patients resistant to cetuximab (13.6%) and in up to 36% of xenopatients in the subset of quadruple wild-type (KRAS, NRAS, BRAF and PIK3CA) in which cetuximab was ineffective [16]. HER3 mutations have been found in approximately 11% of colon and gastric cancers and they can limit the responsiveness to EGFR inhibitors, even if HER2 is not amplified [19].

Secondary resistance could also be associated with EGFR mutations, which may occur in approximately
20% of patients treated with cetuximab and 1% of patients treated with panitumumab [51]. The most frequent mutations recognized in patients, namely S492R, R451C and K467T, involved the extracellular domain of the receptor and, except for R451C, are located in the cetuximab-binding region, thus preventing the binding of cetuximab to the receptor, but not of panitumumab [17, 18]. CRC cell lines made resistant to cetuximab can present other EGFR variants such as S464L, G465R and I491M [17], whereas mutations in the EGFR kinase domain at codons 714 and 794 were identified in circulating DNA of patients with secondary resistance [44].

MET, a tyrosine kinase receptor activated by hepatocyte growth factor and involved in several cell processes, could be amplified in approximately 2% of mCRCs [52]. MET amplification is responsible for the development of distant metastases and it is associated with poor outcomes; moreover, MET amplification has been recognized as a mechanism of both primary and acquired resistance to anti-EGFR therapies in patients with KRAS wild-type mCRC. In cetuximab-resistant xenopatients which are wild-type for RAS, BRAF, PIK3CA and HER2 the rate of MET amplification is increased (12.5%) [20].

Strategies to overcome resistance to anti-EGFR mAbs

As earlier data showed that resistance was mainly linked to the presence of mutations in the EGFR and its pathway, many studies, both at preclinical and clinical levels, were performed utilising inhibitors of proteins situated in this signalling pathway.

One of the first approaches utilized to overcome resistance to anti-EGFR mAbs was based on the use of MEK1/2 inhibitors, as direct inhibition of KRAS showed an absence of clinical efficacy [7]. Several MEK1/2 inhibitors have been studied but the first generation of these compounds did not progress to clinical trials due to their severe toxicity or undesirable pharmaceutical properties [53].

Selumetinib, a highly selective and potent uncompetitive oral inhibitor of MEK1/2, was able to prevent ERK1/2-mediated growth factor-independent survival. Preclinical data showed that selumetinib could be effective to overcome resistance to anti-EGFR mAbs in CRC. Yoon et al. [54] provided in vivo evidence of therapeutic strategy to overcome cetuximab resistance due to KRAS mutation using a xenograft tumour model of CRC treated with selumetinib. Other studies evidenced the possibility to combine selumetinib and cetuximab to obtain an inhibition of proliferation in cell lines resistant to cetuximab harbouring KRAS mutations [10]. A phase I study was undertaken to determine the tolerability and pharmacokinetic profiles of the combination of selumetinib and cetuximab, with an expanded cohort in KRAS-mutant CRC. Selumetinib was administered orally at 50 mg daily or 50-75 mg twice daily and cetuximab was administered at standard doses; cycles were repeated every 28 days. In the expanded cohort, five patients showed stable disease (SD), while nine patients had progression disease (PD) among fourteen evaluable patients [55]. Another highly selective, orally bioavailable, small-molecule inhibitor of the MEK1/2 kinases is pimasertib. Several studies have demonstrated that pimasertib possessed potent antitumor activity either alone or in combination with other agents in cell lines and xenograft models [54, 56]. In a phase I study, patients with KRAS mutant mCRC were treated in the second-line setting with FOLFIRI plus pimasertib [57]. Sixteen patients were enrolled, and of the fifteen patients in the efficacy analysis set, nine experienced SD, two had a partial response (PR) and three had PD as their best overall response. SD was maintained for at least 12 weeks in six of nine patients [57].

Thereafter, the importance of BRAF in the onset of resistance in patients treated with anti-EGFR mAbs was evidenced. Also for BRAF, the clinical approach to treat patients harbouring BRAF mutations was initially based on the use of selective BRAF inhibitors such as vemurafenib and dabrafenib. Vemurafenib and dabrafenib are oral BRAF inhibitors, able to inhibit BRAF kinases with activating codon 600 mutations. Vemurafenib is indicated in monotherapy for the treatment of adult patients with unresectable or metastatic melanoma with a BRAF V600 mutation [58]; dabrafenib shows the same indications, but can be administered as monotherapy or in combination with trametinib [59]. In a phase II study, vemurafenib was administered at 960 mg twice daily in monotherapy
in twenty-one patients with chemorefractory mCRC harbouring BRAF mutations. Among these patients, no one achieved complete response (CR), one patient had a confirmed PR, and seven other patients had SD as the best response for at least 8 weeks [60]. Also with dabrafenib the response in CRC was not excellent: in 9 of colorectal patients included in a phase I study, aimed to evaluate safety and tolerability of this drug in patients with incurable solid tumours, only one had a confirmed PR, while seven showed a SD [61]. As many observations indicated that the inhibition of BRAF was able to activate EGFR signalling pathway [62], another approach was to associate EGFR and BRAF inhibitors. Currently, several phase I/II clinical trials are under investigation in which vemurafenib is associated with cetuximab and irinotecan [63], cetuximab and fluoropyrimidines [64] or panitumumab [65]. The results of one of these studies were very recently published, which showed that six of seventeen evaluable patients (35%) achieved a radiographic response by RECIST criteria; these data were consistent with in vivo models, demonstrating tumour regression with the triplet regimen based on cetuximab, irinotecan and vemurafenib [66]. Dabrafenib, instead, is under investigation in a phase I/II clinical trial in combination with trametinib, panitumumab and 5-fluorouracil, in subjects with BRAF-mutation V600E positive CRC and in subjects with CRC with secondary resistance to prior anti-EGFR therapy [67]. Currently, despite the excellent results obtained with vemurafenib and dabrafenib in melanoma with BRAF V600E mutation, we can conclude that in CRC these drugs do not show significant clinical activity in patients with the same mutation.

Di Nicolantonio et al. [38] studied the role of BRAF in CRC response to anti-EGFR mAb therapy. In this paper, in vitro data showed that colorectal cell lines treated with a combination of cetuximab and sorafenib were dramatically sensitive to the treatment, whereas single agents, alone, had limited effects [38]. Hence, the authors hypothesized that the simultaneous inhibition of EGFR and KRAS/ Braf pathway could be a good pharmacological approach. Sorafenib is a multikinase inhibitor, which has demonstrated both anti-proliferative and antiangiogenic properties in vitro and in vivo [68]. Anyway, a phase II study in which sorafenib was administered at 400 mg orally twice daily with intravenous cetuximab weekly in 28-day cycles in KRAS-mutated (codons 12 or 13), evidenced the absence of objective responses in the twenty-six patients treated [69]. As BRAF V600E mutation leads to the activation of the MEK/ERK pathway, inhibitors of these kinases were tested, at a later time, in mCRCs harbouring BRAF and KRAS mutations.

Trametinib, an oral, reversible, highly selective, allosteric inhibitor of MEK1/2 activation and kinase activity, is indicated as monotherapy or in combination with dabrafenib for the treatment of adult patients with unresectable or metastatic melanoma with a BRAF V600 mutation [70]. Infante et al. [71] treated twenty-eight patients with advanced CRC, treatment-refractory KRAS- or BRAF-mutant, with trametinib. In this phase I study, the drug was administered at 2 mg daily for 21 days (every 28 days) but no patients achieved an objective response. Presently, several clinical trials are underway, where trametinib is used in combination with other drugs. An open-label phase I/II multi-centre study was designed with the aim to identify the effect of lapatinib plus trametinib in patients with KRAS mutation and PI3CA wild-type mCRC and other types of solid tumours [72].

Also for another molecular target, PI3K, identified as crucial for the onset of resistance, the selective inhibition or the inhibition of its downstream effectors, such as mTOR or AKT, did not achieve significant clinical results. In an isogenic cellular model, tumour cells carrying oncogenic PIK3CA mutations or PTEN loss of function were sensitive to everolimus, an mTOR inhibitor, except when KRAS or BRAF mutations were concomitantly present [73]. Translating these findings into the clinical setting by performing genetic analysis of tumours from patients treated with everolimus, it was evidenced that cancer patients whose tumours carried PIK3CA mutations in the kinase domain or PTEN loss of function, displayed increased clinical benefit from everolimus treatment, except in the presence of KRAS mutations [73]. A phase I study in patients with advanced solid tumours was carried out in order to identify everolimus doses for cancer treatment. Fifty-five patients were recruited
and clinical benefit was observed in four patients, including one patient with advanced colorectal cancer that achieved a PR [74]. In a phase II clinical trial, everolimus at 70 mg weekly or 10 mg daily was well tolerated but did not confer meaningful efficacy in heavily pre-treated patients with mCRC [75]. Currently, several trials evaluating combinations of PI3K/mTOR with MEK inhibitors are ongoing. A phase I study tested a combination of two experimental drugs, pimasertib and SAR245409, a PI3K/mTOR inhibitor, in the treatment of locally advanced or metastatic solid tumours. Fifty-three patients were enrolled and among the sixteen CRC recruited, four PRs were evidenced (1 patient was KRAS mutated) [76, 77]. Another open-label, dose-finding, phase Ib clinical trial was designed to evaluate the effects of the orally administered PI3K/mTOR inhibitor BEZ235 in combination with the MEK1/2 inhibitor MEK162 [78].

Another possible strategy to overcome cetuximab resistance in CRC could be the administration of anti-EGFR/HER2 drugs such as the small molecule inhibitor lapatinib or the monoclonal antibody pertuzumab. Bertotti et al. [16], published a study in which xenopatients with cetuximab-resistant, quadruple-negative, HER-2-amplified mCRC were treated with lapatinib and cetuximab or pertuzumab. Based on the positive results obtained in this study, the HERACLES trial was designed. This was a multicentre open-label phase II trial in which patients with KRAS exon 2 (codons 12 and 13) wild-type and HER2-amplified mCRC resistant to standard therapies, including anti-EGFR mAbs, were recruited to evaluate the RR of trastuzumab in association with lapatinib or pertuzumab.Recently, the results of the association of trastuzumab and lapatinib were published; eight of the twenty-seven enrolled patients achieved an objective response, one patient achieved a CR and seven PRs; twelve patients had SD. Moreover, the combination was well tolerated, as no grade 4 or 5 adverse events were reported [79].

Troiani et al. [80], unravelled the role of MET in mediating cetuximab resistance in colorectal cancer cells, suggesting that the interaction of EGFR and MET was responsible for the overexpression of TGF-α, a specific EGFR ligand. They utilized a selective MET inhibitor, PHA665752, showing its ability to inhibit cell growth, proliferation, survival signals and impaired cancer cell migration. These data suggest that EGFR and MET inhibition could represent a strategy to overcome cetuximab resistance in CRC patients. Moreover, in a mouse CRC xenograft model, cabozantinib, a c-MET and VEGFR2 inhibitor, was used to evaluate its effect on in vivo growth of tumours and angiogenesis [81], underlining again that MET inhibition could be a potential strategy in the treatment of resistant CRC.

The results obtained with almost all of the kinase inhibitors surely are interesting but, till now, without a significant clinical impact. More interesting results were obtained with regorafenib. Regorafenib is a low molecular weight, orally available, inhibitor of multiple protein kinases, including kinases involved in tumour angiogenesis (VEGFR1, -2, -3, TIE2), oncogenesis (KIT, RET, RAF-1, BRAF, BRAFV600E), and the tumour microenvironment (PDGFR, FGFR) [82]. In preclinical studies, regorafenib has demonstrated antitumor activity in a broad spectrum of tumour models including colorectal tumour models, which is mediated both by its antiangiogenic and antiproliferative effects. Regorafenib is indicated for the treatment of adult patients with mCRC who have been previously treated with, or are not considered candidates for, available therapies. These include fluoropyrimidine-based chemotherapy, an anti-VEGF therapy and an anti-EGFR therapy [82]. The first-in-man phase I clinical trials of regorafenib assessed the preliminary evidence of antitumor activity of this drug in CRC [83]. Hence the trial was expanded and focused on CRC. 38 patients previously heavily treated for CRC were recruited, and received oral regorafenib at 60–220 mg daily (160 mg daily in the extension cohort) for 21 days and 7 days of no treatment. Among the twenty-seven patients evaluable for response, one achieved PR and nineteen had SD [84]. In a phase Ib trial, forty-five patients were treated with regorafenib (160 mg daily) in combination with FOLFOX or FOLFIRI as first- or second-line treatment of colorectal cancer. Among the thirty-eight patients evaluable for tumour response, seven achieved a PR, while twenty-six showed SD as the best response [85]. Finally, an international, multicentre, randomised, placebo-controlled, phase III trial dissected the efficacy of regorafenib in monotherapy for previously treated mCRC (CORRECT) [86]. 760 patients were
randomized to receive regorafenib (505 patients; 160 mg regorafenib daily) or placebo (n = 255) for the first 3 weeks of each 4-week cycle until disease progression, death, or unacceptable toxic effects. PR or SD was achieved in 207 (41%) of the 505 patients assigned to regorafenib and 38 (15%) patients assigned to placebo. Five patients assigned to regorafenib and one patient assigned to placebo had a PR and no patients had a CR. This study showed for the first time an overall survival benefit of a small molecule kinase inhibitor in patients with mCRC refractory to treatment [86].

Currently, several phase I/III trials are ongoing with the aim to dissect the role of regorafenib in patients with mCRC who have progressed after standard therapy [87] or in combination with others chemotherapy regimens such as FOLFIRI [88], cetuximab [89-90] or panitumumab [91].

CONCLUSION

Although the molecular and clinical advancement over the past few years have allowed to increase the PFS and OS in patients with mCRC, the onset of resistance to anti-EGFR mAb therapy remains one of the main clinical problems. In general, selective inhibition of a single kinase is not a successful strategy to treat resistant patients, maybe because these very selective drugs are effective only in a small subset of patients. In fact, regorafenib, an inhibitor of multiple protein kinases, was the only drug able to induce a response in a high percentage of patients when administered in monotherapy [86]. The combination of two or more drugs in complex chemotherapy schemes seems to be a promising approach after the first line of treatment. One of the most exciting strategies that is emerging is undoubtedly the possibility to target HER2, with both kinase inhibitors and mAbs [79]. In conclusion, the enhancement of genetic and biological knowledge to select the right therapy for each patient continues to be our aim.

CONFLICT OF INTEREST STATEMENT

No potential conflicts of interest to disclose.

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