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REVIEW

Present and future of metastatic colorectal cancer treatment: A review of new candidate targets

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Abstract

In the last two decades, great efforts have been made in the treatment of metastatic colorectal cancer (mCRC) due to the approval of new target agents for cytotoxic drugs. Unfortunately, a large percentage of patients present with metastasis at the time of diagnosis or relapse after a few months. The complex molecular heterogeneity of this disease is not completely understood; to date, there is a lack of predictive biomarkers that can be used to select subsets of patients who may respond to target drugs. Only the *RAS*-mutation status is used to predict resistance to anti-epidermal growth factor receptor agents in patients with mCRC. In this review, we describe approved targeted therapies for the management of metastatic mCRC and discuss new candidate targets on the horizon.

Key words: Novel biomarkers; Monoclonal antibodies; Resistance; Mutation; RAS; Target therapy; Metastatic colorectal cancer

Core tip: Colorectal cancer is a heterogeneous disease characterized by several molecular alterations that affect genes implicated in cancer development. The discovery of novel biomarkers, together with a better understand of the complex biology of the disease, is essential to identify patients who will most likely benefit from personalized treatment.

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INTRODUCTION

Colorectal cancer (CRC) is the third most-diagnosed cancer in Europe and the United States, and 30% of patients with CRC present with a metastatic disease[1,2]. In past decades, substantial progress has been made in the development of new treatment options, which have radically changed the median overall survival (OS) of these patients. The mainstay of metastatic CRC (mCRC) treatment remains the use of cytotoxic agents, as well as irinotecan or oxaliplatin, which results in an average survival of 18 mo when combined with 5-FU and leucovorin or capecitabine (FOLFIRI/FOLFOX or CAPIRI/CAPOX regimens). The addition of targeted therapy has markedly improved the OS of patients with mCRC, which ranges from 22 to 29 mo[3]. Despite the dramatic improvement in survival, after few months of therapy with anti-epidermal growth factor receptor (EGFR) and anti-vascular endothelial growth factor (VEGFR) antibodies, mCRC patients stop responding to treatment due to intrinsic and acquired resistance to the targeted agents[4]. Recent findings in molecular biology and the ability to collect information from large patient databases have improved our understanding of the genetic evolution of this disease. Specifically, CRC is a heterogeneous disease with different molecular landscapes that reflect histopathological and clinical information. Four different subgroups of CRC have been identified, and each subgroup is associated with different patient outcomes (Figure 1). In this review, we summarise the currently approved treatments for CRC and discuss new targets that are on the horizon.

ADJUVANT SETTING

The use of adjuvant chemotherapy with 5-FU-based regimens is considered the standard of care for stage Ⅲ and stage Ⅱ high-risk CRC and benefits these categories of patients[5]. Moreover, the recent CRC classification, based on distinct molecular phenotypes, has identified a new biomarker that can be used to select patients with high-risk stage Ⅱ colon cancer: mismatch repair (MMR) deficiency. The main function of the MMR system is to identify and repair the mismatches that occur during DNA replication, which ensures genomic conservation and stability. While microsatellite instable (MSI) sporadic CRC constitutes 3%-15% of all CRCs, hereditary CRCs with a high level of MSI (MSI-H) constitute approximately 3%-5% of CRCs and arise exclusively in patients with Lynch syndrome, often called hereditary non-polyposis CRC (HNPCC)[6]. Because MSI has been used to screen for HNPCC, it has garnered increasing interest in the setting of CRC. Moreover, patients with MSI-H stage Ⅱ CRC have a better prognosis but derive minimal benefit from 5-FU adjuvant treatment. However, the addition of targeted therapy to a cytotoxic agent in the adjuvant setting provides no benefit in terms of OS and progression-free survival (PFS)[7,8] due to the low level of neo-angiogenesis and a phenotypical difference in these tumours, which leads to an epithelial to mesenchymal transition that could explain the absence of efficacy with the use of anti-EGFR antibodies.

Despite the good prognosis of early-stage CRC, many patients relapse during or a few months after the completion of treatment. Thus, better tools for molecular selection and new biomarkers are undoubtedly needed.

METASTATIC SETTING

In recent decades, the approval of targeted therapy in association with cytotoxic drugs has significantly improved the OS of patients with mCRC[9-13]. Specifically, vascular endothelial growth factor (VEGF) - and epidermal growth factor receptor (EGFR) - targeting monoclonal antibodies (mAbs) have become integral components of the first-line treatment strategies for mCRC. Moreover, the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) have approved targeted therapies for mCRC in recent years such as the EGFR mAbs cetuximab and panitumumab for use in patients with RAS wild-type tumours; for RAS mutant disease, the VEGF mAb bevacizumab, the anti-VEGF receptor 2 (VEGFR2) mAb ramucirumab, the recombinant fusion protein ziva flibercept, and the oral multikinase inhibitor regorafenib have been approved and are discussed below.

Anti-EGFR antibodies such as aflibercept [a decoy receptor for VEGF-A, VEGF-B and placental growth factor (PIGF)] and ramucirumab (an antibody against VEGFR-2) are effective as monotherapy in previously treated patients and in combination with chemotherapy in the second-line setting, and regorafenib (a multikinase inhibitor) is effective as monotherapy in the refractory setting[14].

Anti-VEGFR drugs

In the field of targeted therapy, blocking angiogenesis

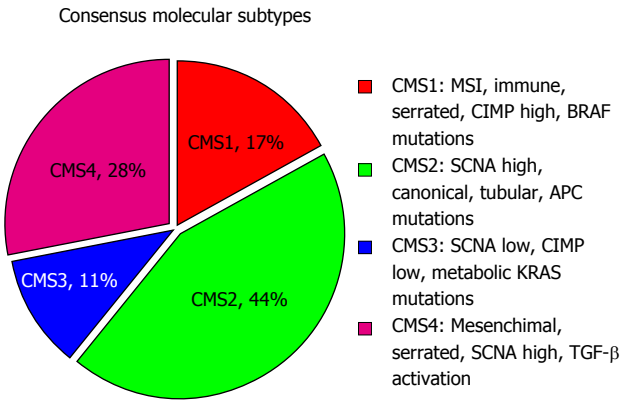

Figure 1 Colorectal cancer consensus gene expression-based subtypes[83]. CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; SCNA: Somatic copy number alterations; BRAF: B-Raf proto-oncogene, serine/threonine kinase; KRAS: Kirsten rat sarcoma viral oncogene; TGF: Transforming growth factor; APC: Adenomatous polyposis coli.

has been considered a fundamental step in mCRC[14]. The deregulation of the VEGF receptor, its cognate cytokines and receptors as well as platelet-derived growth factor receptor has been established to be associated with tumour progression and metastatic spread *in vitro* and *in vivo*[14,15]. To date, the United States FDA and the EMA have approved 3 anti-VEGF agents for the treatment of mCRC.

Bevacizumab is an IgG-1 mAb with a high affinity for soluble VEGF-A that has been tested in early phase Ⅰ and Ⅱ trials[16] and subsequently investigated in phase Ⅲ randomised trials. As a first-line treatment for mCRC in combination with 5-FU/LV/irinotecan or oxaliplatin, bevacizumab has been shown to increase PFS and the response rate (RR)[17].

A recent trial that reported one of the longest survival periods to date investigated the use of the combination of FOLFOXIRI and bevacizumab as a first-line strategy, which resulted in better PFS and RR than FOLFIRI plus bevacizumab[18].

An Eastern Cooperative Oncology Group study (E3200) showed that the median survival for patients treated with FOLFOX4 and bevacizumab was 12.9 mo, whereas that of patients treated with FOLFOX4 or bevacizumab was 10.8 (HR = 0.75, $P < 0.001$) and 10.2 mo, respectively, in the second-line setting[19]. The use of bevacizumab as a maintenance treatment in patients who responded to treatment or present with stable disease upon induction therapy is controversial; however, in the AIO0207 trial, although non-inferiority for bevacizumab alone was demonstrated, the association of bevacizumab with capecitabine, compared to bevacizumab alone, may be the preferable option[20]. Furthermore, the CAIRO3 study showed that initial treatment with capecitabine, oxaliplatin, and bevacizumab (CAPOX-B) and continued with capecitabine and bevacizumab maintenance therapy provided a PFS benefit without compromising quality of life in patients compared with observation alone[21]. Bevacizumab is associated with specific class-related side effects, *e.g.,* hypertension, proteinuria, arterial thrombosis, mucosal bleeding, gastrointestinal perforation and wound-healing problems but does not increase chemotherapy-related side effects[2].

Ziv-aflibercept is a fusion protein that consists of the human extracellular VEGFR domains fused to the Fc portion of human immunoglobulin G1 and work as a trap VEGF-A, VEGF-B and PIGF.

A large phase Ⅲ trial investigating the activity of aflibercept in combination with FOLFIRI found a significant improvement in OS over FOLFIRI combined with placebo in patients with mCRC previously treated with an oxaliplatin-based regimen[22] (HR = 0.817, 95%CI: 0.713-0.937, $P = 0.0032$), with median survival times of 13.50 and 12.06 mo, respectively. Efficacy was maintained with a similar safety profile. Therefore, aflibercept was approved by the EMA after oxaliplatin-based therapy in combination with FOLFIRI.

Ramucirumab (IMC-1121B) is a fully humanized IgG-1 mAb that binds with high affinity to the extracellular VEGF-binding domain of VEGFR-2 and blocks VEGF ligands from binding this site and activating the receptor. The inhibition of VEGF-stimulated VEGFR-2 activation endows ramucirumab significant antitumour activity in a range of malignancies in *in vivo* models as a single agent or in combination with other drugs. Based on the RAISE trial, which enrolled 1072 patients (536 in each group) and randomized them to receive either ramucirumab or placebo, the EMA and FDA approved ramucirumab in the second-line setting for patients whose disease has progressed on first-line bevacizumab-, oxaliplatin- and fluoropyrimidine-containing regimens[23]. The median OS, *i.e.,* the primary endpoint, was 13.3 mo (95%CI: 12.4-14.5) for patients in the ramucirumab group *vs* 11.7 mo (10.8-12.7) for the placebo group (HR = 0.844, 95%CI: 0.730-0.976; log-rank $P = 0.0219$). PFS was significantly improved in patients who received the combination compared to placebo (median PFS 5.7 *vs* 4.5 mo, HR = 0.79, 95%CI: 0.70-0.90, $P < 0.001$).

Anti-EGFR drugs

The EGFR signalling pathway has been identified as a major driver of the development and progression of CRC[24,25].

Several ligands, such as EGF, amphiregulin, or epiregulin, bind specific extracellular domains of the EGFR, which activates an intracellular signalling cascade *via* different signalling pathways. The mitogen-activated protein kinase (MAPK) pathway, comprising RAS-RAF-MEK-ERK and the PI3K-AKT- (PTEN)-mTOR pathway are the main downstream effectors of EGFR implicated in different processes, such as cancer initiation, invasion, angiogenesis, inhibition of apoptosis and metastasis[24,25]. Therefore, EGFR is considered one of the most important targets in CRC treatment.

The anti-EGFR antibodies cetuximab (an IgG1 recombinant human/mouse chimeric anti-EGFR mAb) and panitumumab (an IgG2κ recombinant, fully human anti-EGFR mAb) have been investigated in several phase Ⅲ clinical trials and showed efficacy in terms of PFS, OS, RR, and quality of life among different lines of treatment[26,27]. These antibodies have been shown to prolong survival in patients with mCRC when introduced as monotherapy or in combination with irinotecan in a refractory population[10].

Despite the demonstrated strong benefit, cetuximab and panitumumab achieved a RR of only 10% when used in unselected patients[17]. This result is in concordance with the presence of genetic alterations in EGFR, in the downstream proteins of the EGFR pathway or in other receptor tyrosine kinases (RTKs) that cause resistance to these anti-EGFR antibodies, a phenomenon called primary or intrinsic resistance[28].

Moreover, genetic alterations induced by blocking EGFR cause the positive selection of independent clones or treatment-induced mutagenesis and result in tumour-intrinsic genomic instability that is related to the development of an acquired or secondary resistance to anti-EGFR therapy, emerging at treatment failure[29]. Furthermore, the overall scenario is complicated by the coexistence of different molecular alterations in distinct tumour lesions (inter-metastases heterogeneity) or within different regions of the same lesion (intratumour heterogeneity)[30].

In the era of "personalized treatment" both clinical and molecular data have shown that patients with metastatic CRC have a heterogeneous prognosis and response to treatment. Unfortunately, the complex molecular landscape of the tumour remains incompletely understood, and predictive biomarkers to select patients who may benefit from target drugs are lacking.

Predictive value of RAS

The *RAS* gene is often mutated in mCRC, and the most common of these mutations is Kirsten rat sarcoma viral oncogene (*KRAS*). The *KRAS* gene is mutated in approximately 40% of CRCs; specifically, somatic single-nucleotide point mutations occur in codons 12 and 13 of exon 2 of the *KRAS* gene and in a small percentage in codons 61 and leading to a constitutively activation of the MAPK pathway[31]. Because cetuximab and panitumumab demonstrated a lack of benefit when used as monotherapies for patients with chemorefractory mCRC, researchers investigate the negative impacts of these drugs. Retrospective analyses from randomized controlled trials established that these mutations can predict resistance to anti-EGFR mAb treatment in mCRC. Therefore, the EMA and FDA initially only approved cetuximab and panitumumab for the treatment of patients with *KRAS* exon 2 wild-type tumours[32].

In recent years, several biomarkers in addition to *KRAS* exon 2 mutations were identified to be involved in resistance to anti-EGFR therapy and help to determine a more appropriate patients' selection. Specifically, the presence of other mutations in *KRAS* (exon 3, codons 59/61 and exon 4, codons 117/146) and *NRAS* (exon 2, 3 and 4) correlates with a loss of efficacy of anti-EGFR antibodies, and retrospective and prospective trials have underlined the importance of a selection of patients based on RAS status. Notably, a retrospective analysis of the PRIME trial assessed the "expanded RAS" (*KRAS* and *NRAS*) status and demonstrated the efficacy of the panitumumab plus FOLFOX4 regimen in terms of the objective RR (ORR), PFS and OS compared with chemotherapy alone as a first-line treatment for *RAS WT* mCRC[13]. In other phase Ⅱ and phase Ⅲ trial analyses, the range of mutated patients changed from almost 15% (exon 2 *KRAS* mutation) to 53% (all RAS)[33-36], showing that this population is refractory to anti-EGFR therapy.

Results from a study published by our group, the phase Ⅱ CAPRI trial, demonstrated that patients with mCRC continued to benefit from cetuximab, even after they became refractory to FOLFIRI backbone chemotherapy[37]. After progression on a first-line treatment consisting of FOLFIRI plus cetuximab, patients were randomized to receive FOLFOX alone or in combination with cetuximab. The addition of cetuximab improved PFS when patients were appropriately selected for extended RAS assessment as well as two other potential biomarkers, B-Raf proto-oncogene, serine/threonine kinase (BRAF) and Phosphatidylinositol-4,5-Biphosphate-3-Kinase Catalytic Subunit Alpha (PIK3CA). The results from this trial confirmed the lack of efficacy of cetuximab in the subgroup of patients with *KRAS* and *NRAS* mutations[37] and suggest that continuing cetuximab treatment in combination with chemotherapy is effective in patients who have been molecularly selected. However, these results should be validated in randomized phase Ⅲ trials.

The results emerging from the FIRE 3 trial underscore the importance of expanded *RAS* mutational analysis in the selection of patients. Previously, untreated patients with *KRAS* exon 2 wild-type mCRC were randomized to receive FOLFIRI with either cetuximab or bevacizumab. The trial showed EGFR molecular antibodies were superior in the *RAS WT* population in terms of OS, RR, depth of response and early tumour shrinkage, whereas the initial results of this study did not demonstrate a statistical significant difference in terms of PFS or ORR[38].

Furthermore, a systematic review and meta-analysis of nine randomized, controlled trials evaluating EGFR antibody therapy in all lines of mCRC treatment confirmed these observations[31]. Specifically, the analysis showed that patients with tumours without *RAS* mutations had a significantly better treatment outcome with EGFR mAb therapy than patients whose tumours harboured *RAS* mutations.

Taken together, these results highlight the important role of the RAS status as a predictive biomarker in

the management of CRC. Therefore, the EMA and FDA restricted the indication of cetuximab and panitumumab to "all RAS WT" CRC patients in 2013[34,35].

Not all KRAS mutations are considered equal in giving resistance to anti-EGFR therapies[39]. For instance, retrospective analyses from a phase Ⅲ trial and preclinical data demonstrated that the presence of a *KRAS* G13D mutation allows mCRC patients to respond to cetuximab in both first-line and advanced settings[40,41]. Two phase Ⅱ trials investigated the prospective role of KRAS G13D mutation in response to cetuximab. Neither the first one, conducted from Schirripa *et al*[42] and Segelov *et al*[43] or the ICE-CREAM trial observed any response among the treated patients, with cetuximab therapy.

Finally, *KRAS* is amplified in a small percentage of tumours, and this amplification is considered to be responsible for both primary and acquired resistance[44].

New drugs targeting RAS

One of the most common approaches to inhibiting RAS has been the identification of downstream effectors, as well as MEK and PIK3CA. MAPK-ERK pathway is a convergence point where several upstream signalling pathways can be blocked. Specifically, the combination of trametinib (anti-MEK) and palbociclib (anti-CDK4/6) was investigated as a novel treatment approach in a xenograft model derived from patients with *KRAS*-mutant CRC, and the resulting data showed that this treatment was well tolerated and highly efficacious. Nevertheless, a clinical evaluation is necessary to confirm these preclinical data[45,46].

Reovirus Serotype 3 - Dearing Strain (Reolysin®, Oncolytics Biotech Inc., Calgary, AB, Canada) is a naturally occurring, ubiquitous, non-enveloped human reovirus that can replicate in RAS-transformed cells to cause cell lysis, and its role has been investigated in targeting *KRAS* in mCRC. Specifically, a multicentre phase Ⅰ study testing Reolysin in combination with FOLFIRI and bevacizumab in FOLFIRI-naive patients with *KRAS* mCRC is on-going (ClinicalTrials.gov Identifier: NCT01274624)[47].

Other biomarkers of resistance

The identification of genetic determinants of primary resistance to anti-EGFR therapies in CRC, in particular the activation of an alternative pathway, which can bypass EGFR blockade, is important to identify patients who should not be treated with EGFR mAbs[48]. Beyond *RAS*, additional mechanisms of intrinsic resistance have been identified.

BRAF

Despite the recognition of *KRAS/NRAS* mutations as predictors of a lack of response to anti-EGFR antibodies, a considerable percentage of WT *RAS* CRC tumours do not respond to the appropriately selected targeted therapy, which may be due to a downstream effector of the KRAS/NRAS pathway. Such effector is represented by *BRAF*, a serine/threonine protein kinase that is mutated in 12% -15% of patients with mCRC[49]. A $BRAF^{V600E}$ point mutation is the most common alteration and believed to be mutually exclusive with KRAS exon 2 mutations. Nevertheless, data from the CAPRI trial show concurrent other molecular alterations, such as *TP53, KRAS* and *PI3KCA* exon 9 and exon 20 alterations, in 12 of 15 BRAF-mutated samples[50,51]. The $BRAF^{V600E}$ encodes a constitutively active protein, which would account for the lack of blocking EGFR with cetuximab or panitumumab. Accordingly, several clinical trials have highlighted the poor prognostic role of the *BRAF* mutation in patients with mCRC. For example, Prahallad *et al*[52] reported a median OS for patients with *BRAF*-mutant mCRC of 10.4 mo, compared with 34.7 mo for patients with *BRAF* WT tumours. Furthermore, a retrospective analysis showed that two-thirds of *BRAF*-mutant tumours are located on the right side of the colon and associated with a major incidence of peritoneal disease and distant lymph node involvement. Moreover, a sizeable body of literature established the poor prognostic role of the $BRAF^{V600E}$ mutation, which is associated with increased colon cancer mortality[53,54], but its value as a predictive biomarker remains uncertain due to the absence of prospective trials. In a subset analysis of the PRIME trial, the $BRAF^{V600E}$ mutation indicates any prediction of benefit for the addition of panitumumab to FOLFOX in the first-line setting of mCRC. In addition, data from the MRC COIN trial showed that cetuximab was detrimental in patients with the $BRAF^{V600E}$ mutation[13]. A recent meta-analysis of phase Ⅲ trials confirmed this lack of benefit of mAbs in addition to doublet chemotherapy in terms of OS, PFS and ORR[55]. However, standard therapeutic options for this subgroup of patients are limited. Results derived from a subgroup analysis of the TRIBE trial of 28 patients with the $BRAF^{V600E}$ mutation indicated that patients are more likely to respond to an aggressive initial treatment that combines FOLFOXIRI (fluorouracil, leucovorin, oxaliplatin and irinotecan) and bevacizumab (median OS 19.1 mo *vs* 10.8 mo for the FOLFIRI and bevacizumab group), with a hazard ratio for progression of 0.55 in favour of the combination. Given the impressive results obtained in metastatic melanoma, vemurafenib and dabrafenib have been investigated in $BRAF^{V600E}$-mutated mCRC[56,57]. In a phase Ⅱ trial, vemurafenib was tested in previously treated patients with mCRC; unfortunately, the benefit in terms of RR was only 5% compared with the strong clinical activity demonstrated in melanoma tumours[58]. Moreover, *in vitro* experiments showed that mCRC cells do not respond to vemurafenib due to the persistent activation of the EGFR and the consequent dimerization of BRAF, which suggests that the EGFR signal should be blocked downstream. Current studies are focusing on the dual blockade of BRAF and EGFR or the downstream pathway[59]. According to initial

results, combining the BRAF inhibitor vemurafenib with the EGFR inhibitor panitumumab has been safe, but response has been modest. Nevertheless, ERK inhibitors, which are thought to suppress MAPK activity and overcome resistance to RAF inhibitors, may constitute a treatment strategy.

In this regard, the combination of anti-EGFR antibodies, BRAF inhibitors and MEK inhibitors has recently been investigated and is producing very interesting results[57].

Patients with metastatic CRC and tumours harbouring the BRAF^{V600E} mutation who received triple therapy with dabrafenib (Tafinlar), trametinib (Mekinist), and panitumumab (Vectibix) showed an improved best overall response and prolonged progression-free survival compared to patients who received panitumumab plus either dabrafenib or trametinib, according to results reported by Van Cutsem *et al*[60] at the 2016 European Society for Medical Oncology (ESMO) Congress in Copenhagen (Abstract 455O). Based on preclinical evidence showing that the addition of irinotecan to vemurafenib and cetuximab reduced tumour size, improved response rate and prolonged OS in xenograft models of BRAF^{V600E} metastatic CRC (Yang *et al*[61]), a phase Ⅱ study of irinotecan and cetuximab with or without vemurafenib in *BRAF* mCRC is currently recruiting patients[62]. Specifically, the trial investigates the activity of vemurafenib plus cetuximab and irinotecan compared to cetuximab plus irinotecan in patients with the BRAF^{V600E} mutation. The triplet had an acceptable toxicity profile and may be effective for patients with the BRAF^{V600E} mutation, but the need for a novel therapeutic agent remains.

PI3KCA

In addition to *NRAS/KRAS* and *BRAF* mutations, other predictive biomarkers also indicate resistance to cetuximab/panitumumab[63]. For example, *PIK3CA/AKT/mTOR* signalling pathway is associated with several RTKs, including EGFR. Approximately 10%-20% of CRCs harbour activating mutations of *PIK3CA*, which primarily occur in exons 9 and 20 and are responsible of lack of response to anti-EGFR therapy[64,65]. Accordingly, a retrospective analysis of 110 patients with mCRC treated with mAbs demonstrated the correlation between *PI3KCA* mutations and resistance to treatment with cetuximab or panitumumab in the subset of *KRAS WT* tumours[66].

The precise predictive role of *PI3KCA* mutations is not clear due to the concomitant presence of *KRAS* or *BRAF* mutations and their low incidence, especially exon 20 mutations. However, a large retrospective analysis of 1022 tumour samples of patients treated with cetuximab yielded two main results: only *PIK3CA* exon 20 mutations predict of a lack of response to cetuximab in the *KRAS WT* subpopulation; *PIK3CA* exon 9 mutations and *KRAS* mutations were associated, suggesting a secondary role of *PIK3CA* exon 9 mutations in cetuximab resistance[32]. *PIK3CA* mutations have also been identified as mechanisms of secondary resistance in samples from patients who relapse after treatment with EGFR-targeting mAbs[67]. With respect to the role of *PIK3CA* mutations as a prognostic biomarker, colon cancerspecific mortality is increased in patients with *PIK3CA*mutated tumours compared with patients with WT tumours, even if the worse prognosis in WT tumours is associated with both the presence of exon 9 and exon 20 mutations[68].

The PIK3CA signalling pathway may also be activated by the loss of *PTEN*, which is found in 30% of CRCs and associated with a lack of objective tumour response and worse OS in patients with *KRAS WT* tumours treated with a cetuximabbased regimen[69]. Nevertheless, the *PTEN* expression status does not affect clinical practice since its role as a predictive biomarker remains under investigation.

Several studies have investigated the predictive and prognostic roles of PTEN loss; *PTEN* encodes a phosphatase that is involved in the regulation of the intra-cellular levels of phosphatidylinositol-3, 4, 5-trisphosphate and acts as a tumour suppressor by negatively regulating the AKT/PKB signalling pathway[70]. *PTEN* loss in CRC can occur *via* several genetic and epigenetic mechanisms, such as mutations, promoter hypermethylation or 10q23 LOH and promoter hypermethylation, which leads to subsequent AKT hyperphosphorylation and inhibits apoptosis. Several studies have investigated the predictive and prognostic role of *PTEN* loss; however, data on the concordance rate of *PTEN* expression on primary tumours and matched metastases are controversial.

Mao *et al*[71] conducted a meta-analysis of eight studies to investigate the role of PTEN expression in CRC. In all studies, *PTEN* status was detected using immunohistochemistry (IHC) due to the multiple genetic and epigenetic mechanisms leading to a lack of protein function. In one study included in the meta-analysis[72], *PTEN* expression was analysed in 45 pairs of primary tumours and related metastases. The level of concordance reported was 60%, suggesting that *PTEN* loss contributes to tumour heterogeneity by anti-EGFR treatment pressure. Conversely, a more recent study conducted on 70 matched specimens found a high concordance rate of PTEN expression between primary tumours and liver metastases (98%)[73]. However, a large prospective trial should be conducted to confirm the emerging predictive value of PTEN loss using a validated scoring system for IHC.

New potential treatments that were recently investigated include the combination of the mTOR inhibitor everolimus with panitumumab and irinotecan as a first-line regimen for mCRC[74]. Notably, preliminary results derived from the use of lowdose aspirin in patients with *PIK3CA*mutant tumours indicated a benefit in survival due aspirin mediated COX2 inhibition. However, this observation requires further clinical evaluation[75].

Human epidermal growth factor receptor 2/human epidermal growth factor receptor 3

Human epidermal growth factor receptor 2 (HER2) is an oncogenic driver and member of the ERBbB family, which is targeted by trastuzumab antibody in breast and gastric cancer treatment[4]. The activation of this receptor requires heterodimerisation with other ligand-bound receptors of the same family because of the absence of known HER2 ligands. The heterodimer HER2-HER3 represents a powerful activator of intracellular signalling[76].

HER2 has been proposed as a target in CRC due to studies of *RAS/BRAF* wild-type and cetuximab-resistant CRC xenograft models. In the study conducted by Bertotti *et al*[77] the amplification of the *HER2* gene was recognised as a potential mechanism of primary resistance to cetuximab in a quadruple WT population (*KRAS*, *NRAS*, *BRAF*, and *PIK3CA* wild-type).

The authors only observed *HER2* amplification in a small percentage (2%-3%) of genetically unselected patients with CRC. This proportion increased when considering *KRAS WT* patients who are resistant to cetuximab, ranging from 13.6%-36% in the quadruple WT population. To examine the value of HER2 as a positive predictive biomarker, they performed a multi-arm xenotrial using lapatinib, a dual EGFR/HER2 small-molecule inhibitor, and cetuximab or pertuzumab, a mAb directed against the EGFR/HER2 heterodimer. The association resulted active in the subset of cetuximab resistant, quadruple WT HER2-amplified metastatic CRC xenopatients, with achievable implications in the clinical setting. Based on these preclinical results, Siena and colleagues conducted an Italian, phase Ⅱ, proof-of-concept clinical trial assessing the RR of trastuzumab combined with either lapatinib (cohort A) or pertuzumab (cohort B) in *KRAS* exon 2 (codons 12 and 13) WT and HER2 amplified mCRC patients resistant to standard therapies, including anti-EGFRs[78]. The results from cohort A have been recently published, and approximately 5% (48 of 914 patients screened) tumours were found to be HER2 positive. Of the 27 patients enrolled, eight (30%, 95%CI: 14-50) achieved an overall objective response, and the median duration of the response was 38 wk. The median PFS was 21 wk (95%CI: 16-32), whereas the median OS calculated post hoc was 46 wk (95%CI: 33-68). Notably, responses were significantly more common in tumours with a high *HER2* gene copy number, and the PFS was longer in this population. The combination exhibited a good safety profile, with most toxic effects being grade 1 or 2. To date, HER2 is the first druggable target in mCRC that is a good predictor of response to targeted treatments[79]. However, further investigations are needed in earlier lines of therapy, combining treatment with the inhibition of EGFR and HER2-4.

The amplification of *HER2* is not the only molecular alteration that can hyperactivate the HER2 receptor. The overproduction of Heregulin, a HER3 ligand, may also confer resistance to anti-EGFR treatment. Furthermore, a collection of tumour samples and plasma from patients with acquired resistance to cetuximab demonstrated an increased percentage of *HER2* amplification accompanied by higher levels of heregulin in treated patients compared with pretreatment tumour cells[79]. This result corroborates the assumption that a specific driver of primary resistance to anti-EGFR drugs may be implicated in secondary resistance, leading to the constitutive activation of the ERK-MEK pathway. Furthermore, these results underscore that CRC is a complex heterogeneous disease in which the evolution of single clones present at the beginning of treatment confers resistance in more advanced settings of therapy.

HER3, which is mutated in 11% of patients with CRC, may also be a marker of resistance and may limit the responsiveness to EGFR inhibitors, even if *HER2* is not amplified[80]. Moreover, the overexpression of HER3 was associated with a shorter PFS and OS in a subset of patients with metastatic CRC treated with irinotecan and cetuximab as second- or third-line therapy[81].

Moreover, MEHD7945A, a humanized IgG1 mAb with dual anti-HER3/EGFR activity, had a superior activity to monoclonal EGFR targeting agents in multiple xenograft models[82].

Despite the promising results derived from a phase Ⅰ study of patients with pretreated mCRC, a phase Ⅱ randomized trial of MEHD7945A + FOLFIRI *vs* cetuximab + FOLFIRI did not demonstrate the superiority of the experimental arm in patients with KRAS WT mCRC refractory to oxaliplatin[83].

Regarding secondary resistance, more than a molecular driver resulted implicated and *RAS* mutations are the most frequent, with a range of 50%-80% of patients. For instance, mutations that sustain the mechanism of primary resistance can also be validated as mechanisms of acquired resistance, as described above[29]. Genetic alterations were found in the EGFR receptor, preventing the mAb binding, in the downstream effector as well as *BRAF, PI3KCA*, loss of *PTEN* expression and in the activation of parallel pathways such as amplification of *HER2, MET*; all of these are components of EGFR signalling transduction pathway or interact with.

S492R and other EGFR mutations

Mutations in the extracellular domain of EGFR contribute to secondary resistance to cetuximab. Specifically, Montagut *et al*[84] identified a missense mutation in codon 492 (*S492R*) that appeared to hinder cetuximab binding. This allele has never been identified in previously treated tumour samples, which suggests that this alteration is an exclusive marker of secondary resistance. S492R clones continue to respond to panitumumab, which binds a different epitope, and this finding may be translated to the clinic. Specifically, the researchers reported that one patient with the *EGFR S492R* mutation, whose disease progressed after an initial response to cetuximab, achieved an

initial objective response of five months when treated with panitumumab. However, no further analyses were conducted. Furthermore, new mutations in the EGFR extracellular domain (ECD) were identified in two patients with acquired resistance to cetuximab: *R451C* and *K467T*. Tumour samples of 37 patients with mCRC treated or not with cetuximab were analysed, which revealed that these alterations allowed panitumumab binding to a different epitope of the EGFR ECD[67].

The development of new biological techniques has facilitated the identification of new targets in the setting of acquired resistance. For example, analyses of tumour ctDNA in plasma samples collected before and after treatment represent a complete picture of molecular changes in a patient's tumour. Notably, Bettegowda *et al*[85] described mutations in cell-free DNA, such as codons 714 and 794 of the EGFR kinase domain.

The development of new mAbs directed against different epitopes of the ECD of EGFR may be able to overcome resistance to EGFR blockade.

Sym004, which is a new drug composed by a mixture of two recombinant human mouse antibodies that bind non-overlapping epitopes of domain Ⅲ of the EGFR, induces rapid receptor internalization and degradation *via* EGFR cross-linking[67]. The binding region of Sym004 differs from cetuximab and allows the drug to also be used in the presence of mutations in the ECD of the EGFR. The efficacy of this new drug is under investigation in a phase Ⅱ trial as single agent in selected patients with *KRAS WT* CRC progressing on previous cetuximab- or panitumumabbased therapy within 6 mo of trial enrolment[86-88].

MM151 is a mixture of three fully human monoclonal IgG1 antibodies directed towards three different, non-overlapping epitopes of the EGFR, and the activity of MM151 has been demonstrated in preclinical models. Specifically, it improved EGFR pathway inhibition and downstream signalling and enhanced the downregulation of the EGFR and stimulation of the innate immune responses[89]. Notably, MM151 targets regions of the EGFR distinct from those affected by ECD mutations. Based on these preclinical studies, the efficacy of MM151 was explored in the clinical setting, and current phase Ⅰ results show an acceptable safety profile and objective clinical activity in refractory patients with cancer, including those failing cetuximab therapy[90].

FUTURE DIRECTIONS

Immunotherapy

In recent years, cancer immunology has been considered one of the most interesting fields, with substantial results obtained in the treatment of many tumours. For example, blocking the programmed death 1 (PD-1) pathway with antibodies to PD-1 or its ligands has led to remarkable clinical responses in patients with different types of cancer, including melanomas, non-small-cell lung cancer, renal-cell carcinoma, bladder cancer, and Hodgkin's lymphoma[91-93]. Moreover, the expression of PD-1 ligands (PD-L1 or PD-L2) on the surface of tumour cells or immune cells is an important predictive biomarker of response to PD-1 blockade. Unfortunately, CRC seems to present different molecular features, and the rate of response to PD-1 blockade is very low (1 of 33 patients treated), unlike in other malignancies[91].

Because MMR occurs in a small fraction of advanced CRCs and is associated with a prominent lymphocyte infiltrate and a large number of somatic mutations that can be recognized by the patient's own immune system, researchers hypothesized that mismatch repair-deficient tumours are more responsive to PD-1 blockade than mismatch repair-proficient tumours[94]. To this end, Le *et al*[94] conducted a phase Ⅱ study of Pembrolizumab (a humanized anti-PD-1 antibody) in a treatment-refractory stage Ⅳ CRC population. The immune-related objective response rate and immune-related PFS rate were 40% (4 of 10 patients) and 78% (7 of 9 patients) for MSI-H CRCs and 0% (0 of 18 patients) and 11% (2 of 18 patients) for microsatellite stable/proficient MSS CRCs, respectively. Only 1 of 10 patients with MSI-H CRC experienced disease progression, as compared to 11/18 MSS CRC patients. This study provides strong support for MSI testing in advanced CRC. Furthermore, the Checkmate-142 trial investigated the activity of nivolumab (anti-PD-1) as a single agent or in combination with ipilimumab (anti-cytotoxic T-Lymphocyte Antigen 4) in the same subset of patients with mCRC, MSI-H and non-MSI-H, and interim results were presented at the ESMO congress in 2016, which demonstrated an encouraging advantage and tolerable safety profile[95].

Further research is needed to enhance susceptibility of MSS CRCs to immune checkpoint inhibitors. To this end, a phase IB trial presented by Bendell *et al*[96] at the ASCO meeting in 2016 attempted to identify treatments for this subset of patients with MSS disease. Considering the low activity of atezolizumab monotherapy (an engineered antibody that inhibits PD-L1 from binding with its receptors PD-1 and B7.1) in mCRC, MEK-blocking agents have been associated to immune checkpoint inhibitors because they can induce intratumoural T-cell infiltration and enhance PD-L1 activity, as confirmed in a preclinical setting. Cobimetinib plus atezolizumab was well tolerated at the maximum-administered dose in patients with chemorefractory KRAS-mutant mCRC. The combination resulted in a higher clinical response rate in patients with MSS disease than that expected from either cobimetinib or atezolizumab alone. Furthermore, the use of the combination guaranteed an ORR of 17% and a 6-mo OS of 72%, leading to an expansion of the phase IB trial. A phase Ⅲ trial testing the combination of cobimetinib plus atezolizumab *vs* atezolizumab alone or regorafenib alone in patients with unresectable locally advanced or metastatic CRC is

Table 1 Baseline cancer biomarkers shown in preliminary analysis of the Screening Patients for Efficient Clinical Trial Access in advanced colorectal cancer's molecular screening platform

KRAS WT	KRAS exon 2 mutated	KRAS exon 3 and exon 4 mutated
151 of 284 patients (53%) NRAS mutated (KRAS WT) 14 patients (4.9%); 6 patients in exon 2 and 8 patients in exon 3	114 patients in exon 2 (40%) PI3KCA 41 patients (15%), 13 in exon 20 and 28 in exon 9	8 patients in exon 3 (3%), 11 patients in exon 4 (4%) BRAF 18 patients in exon 15 (7%)

KRAS: Kirsten rat sarcoma viral oncogene; BRAF: B-Raf proto-oncogene, serine/threonine kinase.

under investigation[97].

Furthermore, a study by Ahn *et al*[97] presented at the ESMO congress in 2016 defines a subset of patients with stage Ⅱ/Ⅲ CRC who harbour a mutation in the DNA polymerase epsilon (*POLE)* gene and have a better prognosis. These results may be explained by increased immune activity in *POLE*-mutant tumours, including increased $CD8^+$ lymphocyte infiltration, the expression of cytotoxic T cell markers, and effector cytokines, which is similar to that observed MSI cancers.

Although uncommon and found in only 66 of 6448 (1.0%) CRC samples, *POLE* mutations were significantly associated with several patient and tumour factors, including young age, male sex, right-sided location, early disease stage, and the absence of mismatch repair deficiency ($P \leqslant 0.003$ for all associations)[97].

Notably, a multivariable analysis revealed a statistically significant association between the *POLE* mutation and a greatly reduced risk of disease recurrence: HR = 0.34, 95%CI: 0.11-0.76 ($P = 0.006$). This reduced risk was particularly strong in stage II disease and when associated with MSI-H, an accepted biomarker of favourable prognosis in this setting[98].

Entrectinib

Entrectinib is a novel, orally available, selective tyrosine kinase inhibitor targeting tumours that harbour activating alterations in *NTRK1/2/3* (encodingTrkA/TrkB/TrkC), *ROS1* or *ALK*. Entrectinib is the most potent Trk inhibitor in the clinic and free of undesirable off-target activity. This product candidate is in a Phase 2 clinical trial called STARTRK-2, which is the second of the "Studies of Tumour Alterations Responsive to Targeting Receptor Kinases"[99]. The trial is a global, multicentre, open label, potentially registration-enabling Phase 2 clinical trial of entrectinib that utilises a basket design with the screening of patient tumour samples for the relevant targets. Such a basket design takes full advantage of entrectinib, whose preliminary clinical activity is demonstrated across a range of different tumour types and molecular targets.

SPECTAcolor platform

Treatments for patients with cancer are becoming increasingly tailored to the molecular characteristics of the particular patient and disease. Consequently, molecularly characterizing a patient's tumour is now a prerequisite for them to access the appropriate clinical trial for their particular cancer type. Efficient, GCP-conforming and quality-assured molecular screening to identify potential study patients is one of the major challenges for targeted drug development.

The European Organisation for Research and Treatment of Cancer built a collaborative molecular screening platform, Screening Patients for Efficient Clinical Trial Access in advanced CRC's (SPECTAcolor), which provides the necessary infrastructure to screen adult patients with advanced-stage CRC for mutations in CRC biomarkers. SPECTAcolor's successful start has demonstrated its ability to facilitate next-generation cancer clinical trials across 19 clinical centres by recruiting over 500 patients, and results have been presented by SPECTAcolor's coordinator, Dr. Gunnar Folprecht, at the ESMO congress in 2016[100].

The observed frequency of mutations is similar to that observed in previous CRC clinical trials. New therapeutic targets have been identified by gene panel sequencing and allow patients access to specific clinical trials (Table 1).

CONCLUSION

The treatment of CRC has markedly changed in recent years due to the development of new predictive biomarkers that facilitate optimized, tailored therapy. The discovery of new biologic techniques, such as the liquid biopsy approach, elucidate the increasingly complex heterogeneity of this disease and can be used to monitor minimal residual disease, track tumour clonal evolution and design novel therapeutic strategies to overcome the emergence of drug resistance. Despite this exceptional progress, a large subset of patients continues to be unresponsiveness. In the immediate future, further clinical investigations, such as clinical trials, are needed to guarantee to all patients a genetically determined treatment strategy.

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