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Challenging chemoresistant metastatic colorectal cancer: therapeutic strategies from the clinic and from the laboratory

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As survival has improved for patients with metastatic colorectal cancer (mCRC), there is an increasing need for effective and well-tolerated third-line and subsequent-lines of treatment. Despite recent advances with the development of new-targeted therapies in this setting, there remains an unmet need to exploit oncogenic drivers of colorectal cancer and overcome acquired resistance. Potential treatment strategies include revisiting old targets such as human epidermal growth factor receptor 2, RAS, and BRAF and investigating new targets such as c-MET, the PI3 kinase, and Wnt pathways, and also the use of immune-checkpoint inhibitors. Here, we review recent phase III trials exploring approved agents, early trials investigating new drugs for chemorefractory mCRC, and the potential of capturing tumour dynamics during its evolution by liquid biopsy analysis.

Key words: colorectal cancer, metastatic, liquid biopsy, targeted therapies, ctDNA, circulating tumour cells

introduction

As survival has improved for patients with metastatic colorectal cancer (mCRC), there is an increasing need for effective and well-tolerated therapies in third-line and subsequent-lines of treatment. Conventional agents such as capecitabine, mitomycin C, and gemcitabine are less effective in this setting, while rechallenge with oxaliplatin or epidermal growth factor receptor (EGFR)-targeted therapy may benefit some patients [1]. The options for treating refractory mCRC have expanded with the recent introduction of regorafenib and TAS-102, both of which demonstrated improved survival in placebo-controlled phase III trials [2, 3]. Regorafenib, a multikinase inhibitor, is indicated for the treatment of mCRC in patients who have previously received all standard systemic anticancer treatments. TAS-102 is an oral agent consisting of trifluridine combined with tipiracil hydrochloride to improve bioavailability. This agent is currently approved in the United States for the treatment of patients who previously received fluoropyrimidine, oxaliplatin- and irinotecan-based chemotherapy, an anti-vascular endothelial growth factor receptor (VEGFR) biological therapy, and if *RAS* wild-type, an anti-EGFR therapy and in Japan for unresectable advanced or recurrent CRC (only if refractory to standard therapies). In addition, TAS-102 recently received a positive opinion by the Committee for Medical Products for Human Use (CHMP) from European authorities. Despite these advances, there remains an unmet need for new therapies to exploit oncogenic drivers of CRC and overcome resistance. Insights from genetic studies have provided the impetus for efforts to target key signalling pathways, but have also led researchers to revisit established targets. In addition, individualized treatment is required to address the complex molecular biology of CRC [4], in which tumour heterogeneity is prominent at both diagnosis and metastasis, while genomic instability and acquired resistance emphasize the need for ongoing monitoring of the tumour genotype.

This review outlines current and future directions in chemorefractory mCRC, including recent phase III trials, new approaches to targeted therapy, and the potential of liquid biopsy to guide treatment in this setting.

available options for treating refractory mCRC: where do we stand?

Until 2013, European patients with mCRC progressing on standard treatments (i.e. 5-FU, oxaliplatin, irinotecan, bevacizumab,

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and EGFR antagonists) had no further therapeutic options supported by robust evidence from randomized trials. Meanwhile, the use of existing standard treatments increasingly led to a new and challenging situation of patients having disease progression but maintaining good performance status. Consequently, clinicians were facing cancers resistant to all available agents in patients desiring further treatment. Within this context of a clear unmet need for new treatment options, the randomized (in a 2:1 ratio), double-blind placebo-controlled, phase III CORRECT trial of regorafenib was conducted in 760 patients with progressive mCRC after last standard therapy [2]. Regorafenib is an oral multikinase inhibitor targeting both angiogenic and stromal tyrosine kinases, including human VEGFR2, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE-2), fibroblast growth factor receptor 1, platelet-derived growth factor receptor, and oncogenic kinases such as KIT, RET, and BRAF [5]. Study results were reported in 2013, with prolongation of the median overall survival (OS) being observed in regorafenib recipients with a risk reduction in death by 23% when compared with recipients of best-supportive care alone [median 6.4 months for regorafenib versus 5.0 for placebo; hazard ratio (HR) = 0.77; 95% confidence interval (CI) 0.64–0.94; one-sided $P = 0.0052$] [2]. In addition, risk of progression was reduced by half with regorafenib with a median progression-free survival (PFS) of 1.9 months for regorafenib and 1.7 months for placebo; HR = 0.49; 95% CI 0.42–0.58; $P < 0.0001$). Of note, patients were heavily pre-treated, with nearly 50% having received ≥4 prior therapies for metastatic disease. A survival benefit was also reported for regorafenib in Asian patients, with a median OS of 8.8 months with regorafenib versus 6.3 months for placebo (HR = 0.55; 95% CI 0.40–0.77; one-sided $P = 0.00016$) in the double-blind, placebo-controlled, phase III CONCUR trial (randomization 2:1 ratio), which included 204 patients who had received ≥2 previous treatment lines for metastatic disease [6]. These results supported the findings of a *post hoc* analysis of the CORRECT trial, in which regorafenib had similar efficacy in Japanese and non-Japanese subpopulations (Japanese subpopulation: HR = 0.81; 95% CI 0.43–1.51; non-Japanese subpopulation: HR = 0.77; 95% CI 0.62–0.94) [7].

Regorafenib therapy in mCRC was further characterized in the phase IIIb CONSIGN trial, an open-label, expanded-access study conducted in 2872 patients, of whom 96% had received ≥2 prior regimens for metastatic disease [7]. The primary objective was to better characterize the safety profile of this agent. The duration of therapy was 0–30 (median 2.5) months, and the median PFS was 2.7 (95% CI 2.6–2.7) months. Treatment-related grade ≥3 adverse events were reported by 57% of patients. The most common (>5%) grade ≥3 treatment-related AEs were hypertension (15%), hand–foot skin reaction (14%), and fatigue (13%). Furthermore, the safety profile was consistent with previous phase III regorafenib trials in mCRC [2, 6].

A new agent, TAS-102, has also shown a significant OS benefit in refractory mCRC. TAS-102 is an oral drug consisting of trifluridine (FTD), a reversible inhibitor that binds to the active site of thymidylate synthase. Thymidylate synthase plus tipiracil hydrochloride (TPI) improves the bioavailability of FTD by inhibiting its catabolism by thymidine phosphorylase (TP) [8]. TAS-102 was tested over placebo in the multinational, randomized (2:1 ratio), double-blind, placebo-controlled phase III RECOURSE trial which included 800 patients with mCRC who had received ≥2 prior standard chemotherapy regimens [3]. The median OS was 7.1 months with TAS-102 versus 5.3 months with placebo (HR = 0.68; 95% CI 0.58–0.81; $P < 0.001$). On the basis of the results of the RECOURSE trial, TAS-102 was approved by the US Food and Drug Administration (FDA) in September 2015 for use in patients with mCRC previously treated with standard chemotherapy and biological therapy. In Japan, it is approved for the treatment of unresectable, advanced, or recurrent CRC.

Regorafenib, which received a positive opinion by the CHMP from the European Medicines Agency (EMA), is recommended in the European Society of Medical Oncology (ESMO) and US National Comprehensive Cancer Network (NCCN) guidelines (V2.2016) as a standard option for second-line therapy and beyond in mCRC, and TAS-102 is included in NCCN guidelines only (V2.2016) [9, 10]. However, although improvements in OS are being observed in these settings, a significant need remains for patients with refractory mCRC.

'old' versus 'new' targets

Although most therapeutic development for mCRC in the chemorefractory setting focuses on new targets and/or more potent agents, reconsideration of established targets has gained importance with the growth of a rational pharmacogenomic approach to drug development. In the following section, we highlight the most promising old targets, which are well-established cancer biomarkers and/or targeted with FDA- or EMA-approved indications in CRC or other histologies, and new targets currently undergoing clinical evaluation as shown in Figure 1. Table 1 also provides additional information on current trials of select targeted agents in mCRC.

old targets revisited by new pharmacogenomic strategies

human epidermal growth factor receptor 2

The human epidermal growth factor receptor 2 (HER2/neu) is a *bona fide* oncogenic driver and the target of trastuzumab in breast and gastric cancers [26, 27], whereas investigations of its role as a prognostic biomarker and therapeutic target in CRC generated conflicting results. Recent data, however, based on newer diagnostic technologies and more pertinent *in vivo* models, are highlighting a renewed role for this 'old' molecular target also in CRC [28]. HER-2/neu expression rates in CRC in the literature range enormously from 1.6% [29] to 47.4% [30], but the sample size of the series evaluated, inclusion of distinct subgroups, and the use of different diagnostic methods and scoring systems may account for this variability [31–34]. In two of the most recent series, the rate of HER2 positivity [immunohistochemistry (IHC) score of 2+/3+, or *HER2* gene amplification by *in situ* hybridization] ranged from 1.6% to 6.3% [29, 35]. In a consensus study aimed at defining CRC-specific criteria for HER2 positivity [36], an archival test cohort ($n = 256$) and a clinical validation cohort ($n = 830$) were tested by a consensus panel of pathologists, showing a clinically sizeable 5% fraction of *KRAS* wild-type CRC patients displaying HER2-positive tumours that were candidates for therapeutic targeting [11].

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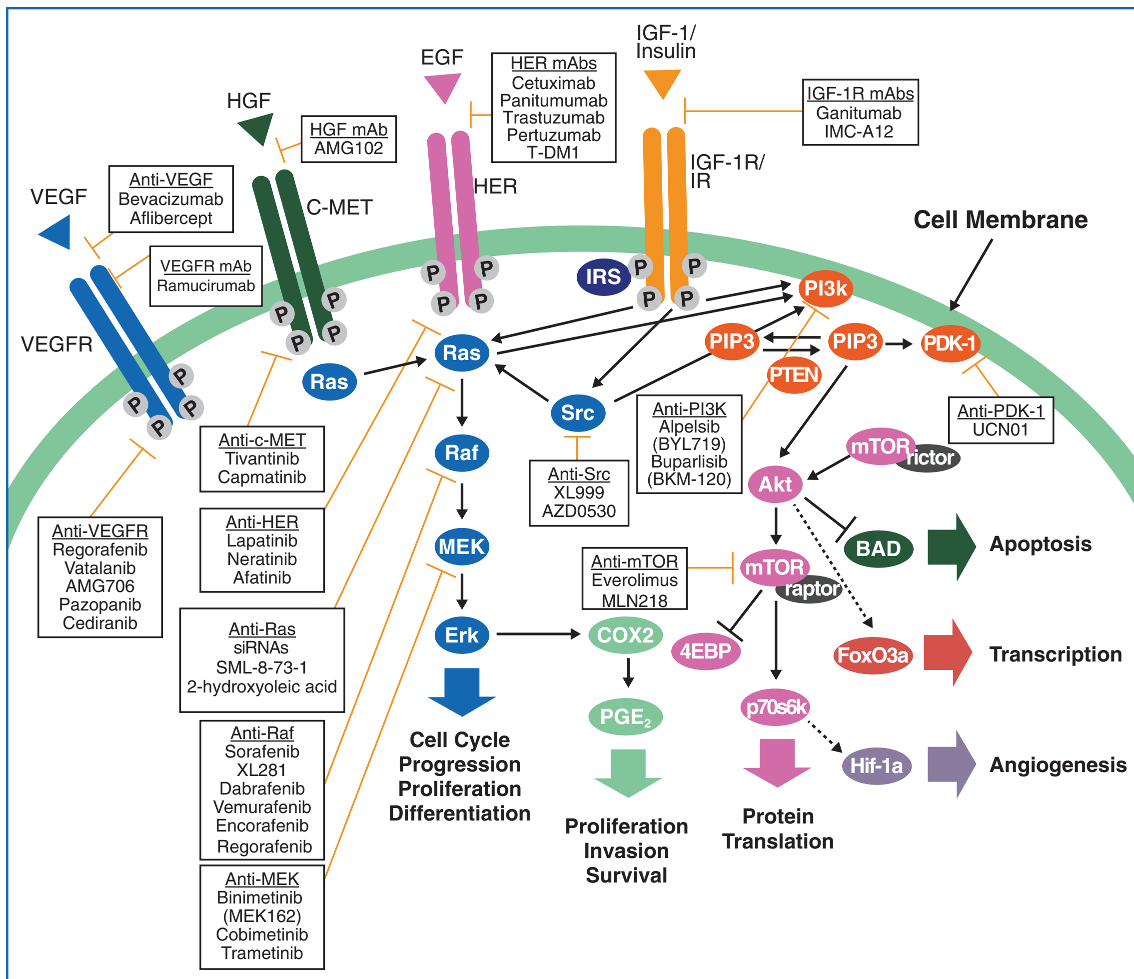

Figure 1. Old and new targets in metastatic colorectal cancer. mAb, monoclonal antibodies; HER, human epidermal growth factor receptor; C-MET, mesenchymal–epithelial transition factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-I; IR, insulin receptor; VEGF, vascular endothelial growth factor.

No association with clinico-pathological parameters emerges from most of these studies, although a trend towards worse survival for HER2-positive patients was noted in a large cohort ($n = 1645$) [29]. A possible association with tumour location in the rectum has also been noted in several studies [29, 35], but retrospective data from the phase 2 EXPERT-C trial, limited to high-risk, locally advanced rectal cancer, showed a 2.8% prevalence of HER2 expression [37]. In addition, Missiaglia et al. [38] reported that distal carcinomas are more likely to be HER2 amplified compared with proximal carcinomas.

Early studies exploiting HER2 as a therapeutic target were problematic due to the flawed study design (e.g. lack of confirmed target amplification and CRC-specific criteria, inadequate sample size, and absence of a rational HER2-targeted combination strategy), and/or poor accrual when enrolment was confined to HER2-overexpressing tumours only. In particular, two trials of trastuzumab combined with chemotherapy in HER2-overexpressing mCRC were prematurely closed due to low accrual, despite evidence of activity [39, 40]. In these studies, partial responses were obtained in 5 of 7 assessable patients treated with trastuzumab plus irinotecan as first- or second-line therapy [39], and in 5 of 21 assessable patients treated with trastuzumab combined with 5-FU plus oxaliplatin in second- or third-line [40] with an HER2 positivity (IHC 2+/3+) rate of 4%–8%. Finally, two cases of mCRC patients with liver metastases who demonstrated clinical response to capecitabine and oxaliplatin (CapeOx) plus lapatinib, a dual HER2/EGFR inhibitor, were reported, but no selection based on HER2 status was carried out [41]. More recently, the ongoing Italian HERACLES trial tested the combination of trastuzumab and lapatinib in patients with HER2-positive and *KRAS* wild-type chemorefractory mCRC. This combination was based on the preclinical activity demonstrated in a molecularly annotated platform of patient-derived xenografts [42]. Of the 914 patients with KRAS exon 2 (codons 12 and 13) wild-type mCRC, 48 were HER2-positive (5%). Of these patients, 27 were eligible for the trial. At a median follow-up of 94 weeks, 8 patients had achieved an objective response, with 1 (4%) achieving a complete response, and 7 (26%) achieving partial responses; 12 (44%) patients had stable disease [11]. These results should be regarded as extraordinary given the heavily pre-treated population of the study (median 5 prior regimens), showing for the first time that there is a genetically defined subpopulation of CRC (5% of *KRAS* WT) with sensitivity to pharmacological blockade of a specific oncogenic

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Table 1. Select targeted therapies in clinical development for colorectal cancer

Therapeutic agent, grouped by target	Molecular target	Phase and trial identifier
HER2 signalling		
Trastuzumab	HER2	Phase II 004-IRCC-10IIS-12 [11]
Pertuzumab	HER2	Phase II 004-IRCC-10IIS-12 [12]
Lapatinib	HER2, EGFR	Phase II 004-IRCC-10IIS-12 [11]
Trastuzumab-emtansine (T-DM1)	HER2	Phase II 004-IRCC-10IIS-12 [12] 011-IRCC-10IIS-15 [13]
Neratinib	HER2, EGFR	Phase II NCT01953926 NCT01960023
Afatinib	HER2, EGFR	Phase II NCT01919879 NCT01152437 [14] NCT02450656
HER2 peptide vaccine	HER2	Phase I NCT01376505
RAS signalling		
2-hydroxyoleic acid	RAS membrane localisation [15]	Phase I/II NCT01792310 [16]
Vemurafenib	BRAF V600 kinase	Phase II NCT02164916 [17]
Dabrafenib	BRAF V600 kinase	Phase II NCT01072175 [18] NCT01750918 [19]
Encorafenib (LGX-818)	BRAF V600 kinase	Phase II NCT01719380 [20] NCT02278133
Trametinib	MEK1, MEK2	Phase II NCT01750918 [19] NCT02230553 NCT02399943
Binimetinib (MEK-162)	MEK1, MEK2	Phase I/II NCT01927341
Cobimetinib (XL-518, GDC-0973)	MEK1, MEK2	Phase I NCT01988896 NCT02457793
MET signalling		
Tivantinib (ARQ-197)	c-MET	Phase II NCT01892527 NCT01075048 [21]
Capmatinib (INC-280)	c-MET	Phase Ib NCT02205398
Phosphoinositide-3 kinase (PI3K) pathway		
Alpelisib (BYL-719)	PI3K-α	Phase II NCT01719380 [20]
Buparlisib (BKM-120)	Class I PI3K	Phase I/II NCT01591421
Wnt pathway		
PRI-724	Inhibits interaction between β-catenin and CREB-binding protein [22]	Phase II NCT02413853
WNT-974 (LGK-974)	Porcupine [23]	Phase I/II NCT01351103 NCT02278133

Continued

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Table 1. *Continued*

Therapeutic agent, grouped by target	Molecular target	Phase and trial identifier
ETC-159	Porcupine [24]	Phase I NCT02521844
Foxy-5	6-amino-acid peptide fragment that mimics the effects of Wnt-5a to impair migration of epithelial cancer cells [25]	Phase I NCT02020291 NCT02655952
Immune-checkpoint inhibition/immune modifiers		
Pembrolizumab	PD-1	Phase III NCT02563002 (KEYNOTE 177); NCT02460198 (KEYNOTE 164)
Nivolumab	PD-1	Phase II NCT02060188 NCT02335918
AMP-224	PD-1	Phase I NCT02298946
PDR-001	PD-1	Phase I/II NCT02460224
Durvalumab (MEDI-4736)	PD-L1	Phase II NCT02227667
Atezolizumab (MPDL3280A)	PD-L1	Phase II NCT02291289
Ipilimumab	CTLA4	Phase II NCT02060188
Tremelimumab	CTLA4	Phase I (combination therapy) NCT01975831
LAG-525	LAG-3	Phase I/II NCT02460224
Varlilumab	CD27	Phase I/II NCT02335918 NCT01460134

CREB, cAMP response element-binding protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; LAG-3, lymphocyte activation gene-3; MEK, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase; PD-(L), programmed cell death protein (ligand).

product. On the basis of these data, dual-targeted therapy with trastuzumab and lapatinib could be considered as a new standard, chemotherapy-free, regimen for CRC patients with HER2-positive tumours in this setting and possibly earlier lines of treatment.

RAS

Activating mutations in *KRAS* and *NRAS* have been reported in ~40% and 8%–10% of CRCs, respectively [28, 43], causing resistance to anti-EGFR monoclonal antibodies [3, 43, 44] and conferring a negative prognosis [45, 46]. Consequently, *KRAS* and *NRAS* have been pursued since the beginning of molecularly driven therapeutics in oncology, while remaining out of therapeutic reach due to the complex matrix of factors that regulates their functions. Instead, indirect strategies have been tested in an effort to target the RAS protein. Targeting RAS membrane localization using farnesyltransferase inhibitors was an early strategy that aimed to indirectly down-regulate RAS-mutant proteins, but this approach failed due to the existence of redundant mechanisms able to overcome farnesyltransferase inhibition [47]. Following the same strategy, NaCHOleate has entered into clinical experimentation. This drug consists of a lipid-based molecule that causes activation of sphingomyelin synthase, thereby normalizing lipid composition of tumour-cell membranes to a more balanced composition between levels of sphingomyelin, diacylglycerol, and phosphatidyl-ethanolamine found in normal cells. These changes in membrane lipidic composition have been shown to impair RAS anchorage and down-regulate MAPK and PI3K pathways in certain tumour types such as glioma cells [15, 48]. On the basis of this, RAS-mutant CRC patients have been allocated to the ongoing phase I clinical of this compound, although the reported results from the dose-escalation phase seem modest for this particular population. Hence, we may need to wait until the final results from the expansion phase of the study to formulate a solid conclusion in this regard [15].

Novel approaches to targeting RAS include small interference RNAs (siRNAs) directed against messenger RNAs of *RAS*-mutated isoforms, which have been demonstrated to down-regulate RAS-mutant proteins in preclinical models as well as in pancreatic cancer patients harbouring *KRAS* G12D mutation [49, 50]. However, the adverse pharmacokinetic profile of siRNAs

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that need local administration in tumours clearly jeopardizes their use in metastatic CRC. The solution for this problem comes with the use of appropriate delivery systems that allow siRNAs to be administered intravenously. This approach was successfully demonstrated in a phase I trial, which included a substantial number of metastatic CRC patients that explored the feasibility of intravenous administration of siRNA encapsulated in to a polilipidic delivery system that laid the groundwork for RAS targeting at the RNA level in metastatic CRC [51]. Meanwhile, there is renewed interest in the direct inhibition of RAS following the development of small molecules that selectively and irreversibly bind to the G12C mutant form of K-RAS [52, 53]. In addition, a new compound, with tyrosine-kinase inhibitory activity, SML-8-73-1, has demonstrated the ability to inhibit the KRAS G12C mutant in preclinical studies, with clinical studies pending [53].

Other indirect strategies target the principal RAS protein effectors. In this regard, two different approaches have been tested so far: parallel inhibition of the mitogen-activated protein kinases (MAPK) and PI3K/Akt/mTOR pathways; and vertical inhibition aimed at blocking MEK plus the main membrane receptors implicated in RAS pathway activation. Both strategies are based on solid preclinical data [54–56], yet preliminary clinical results are less positive as MAPK–PI3K inhibition had an adverse tolerability profile with modest clinical activity in *RAS*-mutant CRC [57], which does not support further clinical development in this population. Vertical strategies to block RAS signalling derive from the failure of using single-agent MEK inhibitors to indirectly target RAS mutations. Following the poor preliminary results for *RAS*-mutated CRC patients reported in phase I trials exploring MEK inhibitors [58–60], preclinical experimentation demonstrated the existence of regulatory feedback that reactivate MAPK and PI3K pathway signalling upon MEK inhibition through crosstalk with HER family membrane receptors, c-MET, and insulin-like growth-factor-receptor 1 [56, 61–63]. Thus, several clinical trials have been initiated to evaluate MEK inhibition in combination with different membrane receptor inhibitors. A phase I study combining selumetinib plus cetuximab reported two partial responses and two long-lasting stabilizations in *RAS*-mutant CRC; on this basis, results from the expansion cohort for *RAS*-mutant CRC are highly awaited [64]. Furthermore, another trial explored the combination of trametinib with panitumumab in one cohort; however, mCRC patients were not selected according to *RAS* status which diluted the possibility of interpreting results [19]. On the other hand, another phase I trial combining selumetinib plus the anti-IGF1R antibody, cixutumumab, reported a long-lasting stable disease of more than 6 months as best response in the *RAS*-mutant CRC cohort [65]. Furthermore, other phase I trials exploring combinations of MEK inhibitors plus panitumumab (NCT01927341), MEDH7945A, anti-EGFR/HER3 bi-specific antibody (NCT01986166), ganitumab (NCT01562899), or crizotinib (NCT02510001) have results pending.

BRAF

In CRC tumours, mutations leading to constitutive *BRAF* activation have been reported in 47% of hypermutated tumours and 3% of non-hypermutated tumours [28], and ∼5%–10% of CRC tumours overall [66]. These mutations are associated with aggressive tumour behaviour and a correspondingly worse prognosis, including shorter OS [67–70]. The low reported *BRAF* mutation frequency precludes reliable conclusions regarding sensitivity to specific antineoplastic agents. In addition, no differential patterns of response to chemotherapy regimens or anti-angiogenic agents have yet been reported [71, 72], whereas an increasing body of evidence suggests that *BRAF* mutation may confer a weaker benefit from anti-EGFR monoclonal antibodies, or even a detrimental effect [73–76]. Some retrospective studies and a prospective phase II single-arm study support the use of an intensive combination of FOLFOXIRI plus bevacizumab as a preferable option to counteract the poor prognosis of *BRAF*-mutant patients [71, 77, 78]. Nevertheless, the percentage of BRAF-mutant patients eligible for such approach in daily clinical practice may be rather low.

In contrast to the favourable results obtained in melanoma patients [79], initial attempts to target *BRAF*-mutant CRC using the BRAF kinase inhibitor vemurafenib yielded low clinical activity, with a response rate (RR) of 5% and median PFS of 2.1 months [17]. The addition of an MEK inhibitor did not substantially increase efficacy, with an RR of 9% and PFS of 3.5 months reported for the combination ofdabrafenib and trametinib [18]. EGFR expression was subsequently identified as a key cause of resistance to *BRAF* inhibition by means of its interplay with MEK: after *BRAF* blockade, MEK-derived EGFR signalling was shown to reactivate MAPK and/or PI3K signalling, causing early tumour progression [80, 81]. Subsequent phase I/II trials have explored the addition of anti-EGFR monoclonal antibodies to BRAF inhibitors, with varying results, for example, an RR of 10% and PFS of 3.5 months, have been observed for dabrafenib–panitumumab [19]. Triplet regimens have also been explored in an attempt to improve efficacy and delay resistance, with an RR of 26% and PFS of 4.1 months reported for dabrafenib–panitumumab–trametinib [19]; and an RR of 50% and PFS not reported for vemurafenib–cetuximab–irinotecan [82]. However, there are considerable toxicities with these combination regimens, especially diarrhoea and skin reactions. In addition, outcomes remain inferior to those seen in melanoma or other *BRAF*-mutant tumours, such as lung adenocarcinoma or papillary thyroid cancer [83]. The results of a confirmatory phase II trial comparing irinotecan and cetuximab with or without vemurafenib in *BRAF*-mutant mCRC and a phase I/II trial of WNT974, cetuximab, and encorafenib in BRAFV600-mutant mCRC with RNF43 mutations or R-spondin fusions will be of interest to clarify the future role of BRAF inhibition in CRC.

immune-checkpoint inhibitors

In recent years, we have witnessed an intense expansion of immune therapeutics in oncology [84], particularly immune-checkpoint inhibitors: anti-CTLA4 and anti-programmed cell death (ligand) protein-1 (PD-1/PD-L1) monoclonal antibodies. Once again contrasting with favourable results in other tumour types, CRC patients derived little benefit from these agents in the initial clinical trials [85–87]. Some insight was provided by a recent study that identified the tumour mutational load as a predictive biomarker of response to the anti-PD-1 monoclonal antibody, pembrolizumab [88]. The immune-related objective response rate was 40% (4/10 patients) for mismatch repair-deficient mCRC,

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versus 0% (0/18 patients) for mismatch repair-proficient mCRC [88]. These findings strongly suggest a need to expand the identification of CRC tumours with microsatellite instability beyond the localized stages to include patients across the disease spectrum. In addition, there is a need for understanding how to enhance susceptibility to immunotherapies in mismatch repair-proficient tumours. Two ongoing studies are investigating pembrolizumab in a naïve patient population (phase III KEYNOTE 177 study; NCT02563002) and in previously treated advanced CRC (phase II KEYNOTE 164 study; NCT02460198).

novel targets under investigation

membrane receptors: c-MET

Activation of the c-MET receptor is linked to cancer cell survival during stress, and also to treatment resistance [89]. In CRC, *MET* amplification, overexpression, and super-activation have been implicated in chemotherapy resistance [89]; consequently, c-MET inhibition to overcome resistance has been explored as a single agent or in combination with chemotherapy. Results have been inconsistent, in part, due to a lack of reliable biomarkers to identify those patients most likely to respond [21, 90]. More recently, studies exploring the molecular mechanisms underlying resistance to anti-EGFR monoclonal antibodies have identified *MET* amplification as a cause of resistance in 5%–12% of patients progressing on these agents [91]. Current efforts to develop c-MET inhibitors for clinical use are exploring the potential of c-MET inhibitors to overcome resistance to EGFR blockade in patients with proven *MET* amplification or c-MET overexpression after progression on anti-EGFR therapy [90].

cytoplasmic targets: PI3K, Akt and PTEN

Mutations in phosphatidylinositol-45-bisphosphate 3-kinase catalytic sub-unit alpha (*PIK3CA*), phosphoinositide-3-kinase, regulatory sub-unit 1 (*PIK3R1*), and Akt, together with deletion or mutation of phosphatase and tensin homolog (*PTEN*), are molecular alterations that lead to activation of the phosphoinositide 3-kinase (PI3K) pathway in CRC [28, 92–94]. Inhibitors of this pathway have been extensively tested in molecularly selected patients, but, in contrast to findings in other tumour types, results in CRC have been poor [95–97], and development has been halted in this indication. However, recent studies investigating temporal clonal evolution in CRC identified a progressive enrichment of PI3K pathway activation, both as the disease progressed and following treatment, highlighting the importance of this pathway in progression and resistance [98, 99]. These results warrant the exploration of these drugs in combination with standard therapies in selected patients, as part of a strategy to overcome resistance.

R-spondin fusions and RNF43 mutation in the Wnt pathway

Deregulation of the Wnt signalling pathway plays a key role in CRC development, with over 90% of CRC tumours harbouring Wnt pathway mutations, notably inactivation of the adenomatous polyposis coli gene (*APC*) (81% of tumours), and activating mutations of the β-catenin gene (*CTNNB1*) (5%) [28]. Most cases of sporadic CRC result from bi-allelic loss of the tumour suppressor gene *APC*, which has been described as a gatekeeper gene [88]. The loss of APC leads to the formation of stable complexes between β-catenin and the transcription factor TCF4; this critical downstream signalling junction has proven difficult to target [100]. However, a drug that inhibits the interaction between β-catenin and the cAMP response element-binding protein (CREB) binding protein, PRI-724, has completed phase I development [22] and will be evaluated in a randomized phase II trial for mCRC (NCT02413853). PRI-724 is administered as a continuous 7-day infusion.

Furthermore, recent genotyping studies revealed the existence of additional molecular alterations of the Wnt pathway that define a subset of CRCs highly dependent on sustained high Wnt levels; these alterations include R-spondin 2 and 3 fusions and inactivating mutations of the tumour suppressor gene *RNF43* and *ZNFR3* [101–103]. Porcupine, an enzyme required for palmitoylation of Wnt ligands, is another target in the Wnt pathway. WNT974 (formerly LGK974) is a small-molecule porcupine inhibitor that decreased Wnt ligand production and down-regulated the Wnt pathway in preclinical models [23]. WNT974 is currently being assessed in two phase I trials. The first is evaluating WNT974 monotherapy and includes patients with *RNF43* or *ZNRF3* as well as with *RSPO2* or *3* fusions (NCT01351103), while the second is investigating WNT974 as combination therapy in BRAFV600-mutant mCRC with *RNF43* mutations or R-spondin fusions. The results of these trials are eagerly awaited.

capturing the instable tumour genome: the role of liquid biopsy

One of the major challenges in the targeted treatment of mCRC is the instability of the cancer genome. Thus, salvage treatment is frequently introduced after ≥4 lines of treatment, and often ≥30 months after the diagnosis of metastatic disease [2, 104]. In routine clinical use, tumour-tissue biopsies are obtained before the initiation of first-line treatment. The somatic mutation profile is a snapshot of the unstable tumour genome and may alter substantially over time, or due to clonal selection of resistant cells during targeted treatment. Serial biopsies should be carried out to guide treatment decisions, but serial invasive diagnostic techniques are not feasible, thus highlighting a high unmet need for circulating markers.

circulating tumour cells versus circulating cell-free tumour DNA

Technical advances now make it possible to obtain genetic information on tumours from peripheral blood, a minimally invasive approach commonly referred to as a 'liquid biopsy'. To date, two different sources of circulating genetic information can be analysed, namely circulating tumour cells (CTCs) and cell-free tumour DNA (ctDNA). In patients with mCRC, the number of CTCs before and during treatment is a strong independent predictor of PFS and OS [105], and enumeration of CTCs using the CellSearch® system (Janssen Diagnostics, LLC; Raritan, NJ, USA) is FDA-approved for monitoring such patients. Isolation and functional characterization of CTCs offers the potential for further analysis, including protein

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expression, activation of signalling pathways, quantitative RNA analysis, and cytogenetic characterization. Characterization of CTCs may lead to the identification of prognostic, predictive, or pharmacokinetic biomarkers, including markers of drug sensitivity and resistance [106]. For example, KRAS mutation analysis of DNA from CTCs in CRC patients identified a connection between the presence of KRAS mutations in ctDNA and the presence of genetically abnormal circulating cells [107].

Recent technical advances have also demonstrated the possibility of single-cell amplification and sequencing, which allows a detailed genomic characterization of CTCs and could potentially be used to re-evaluate the cancer genome before the introduction of a new treatment in patients with advanced disease [108]. A drawback of this method is that CTCs are rare (estimated at one CTC per 10^6–10^8 normal blood cells) [109]. CTC enrichment methods commonly use an epithelial cell adhesion molecule (EpCAM)-based selection system, which may fail to detect cells that undergo mesenchymal transition [109]. EpCAM-independent isolation systems exist, but are still faced with a high cell-to-cell variability, necessitating isolation of a large number of CTCs to obtain a representative profile of the individual cancer genome [110, 111].

Once isolated, CTCs represent a pure tumour-cell population, which can be analysed or used to study functional cell behaviour. Novel advances in molecular characterization at the single-cell level provide a unique opportunity for longitudinal analysis of clonal evolution over the disease course and during different treatment approaches. However, the bottleneck for CTC studies is their low levels and the fact that they have to be identified and isolated for further analysis.

Fulfilling the requirements of an EpCAM-independent, non-invasive biomarker, ctDNA can also be isolated from plasma, serum, or other body fluids, and additionally may reflect the average genotype of all tumour cells. Serial monitoring of nucleic acids has become established in chronic infectious diseases, including HIV infection and viral hepatitis, as a convenient means of tracking viral activity or treatment response. The concept that quantitative assessment of circulating cell-free DNA correlates with tumour burden was first described in 1977 [112]; however, levels may be affected by non-specific factors such as inflammation, trauma, or benign lesions. Isolation of ctDNA is, therefore, preferred using highly sensitive next-generation sequencing of cell-free DNA to detect tumour-specific somatic mutations. When a tumour-specific mutation is known, Beads, Emulsion, Amplification, and Magnetics (BEAMing) technology may be used to quantitatively analyse ctDNA in plasma. In BEAMing, which allows detection of rare mutant alleles, mutation-specific oligonucleotides are used to coat magnetic beads with emulsion PCR and hybridization and analysis by flow cytometry [113]. Using this technique in patients with CRC, Diehl et al. [113] found that serial ctDNA measurements reliably followed tumour dynamics; the recurrence rate also differed significantly between patients with and without detectable mutant ctDNA at the first post-surgical follow-up ($P = 0.006$).

Other studies have demonstrated concordance of liquid biopsies and tumour-tissue biopsies for molecular characterization of oncogenes such as *RAS*. Thierry et al. [114] prospectively compared results from ctDNA characterization with those of standard tumour-tissue biopsies and found that ctDNA had a sensitivity of 92% and a specificity of 98%, with a net accuracy of 96% for *KRAS* and *BRAF* mutations in mCRC patients. As the tumour genome is unstable, it is tempting to speculate whether the inaccuracy of 4% observed in this study might be caused by inaccurate analysis of a biopsy from a single tumour lesion. As ctDNA represents the average tumour genome with a detection limit of ratios >1:10 000 (0.01%), ctDNA analysis might be the more accurate approach; however, translation of this improved accuracy into better patient selection for treatment with molecularly targeted agents is yet to be demonstrated.

Serial analysis of ctDNA has also been used to predict progression and explore potential mechanisms of resistance during anti-EGFR treatment in patients with *RAS* wild-type CRC. Misale et al. [115] demonstrated that acquired *KRAS* mutations were associated with secondary resistance to EGFR blockade and, further, that *KRAS*-mutant alleles were detectable in the blood of cetuximab-treated patients up to 10 months before radiographic evidence of disease progression. A second study found that patients who relapsed on anti-EGFR treatment developed one or more mutations in genes involved in the MAPK pathway, most frequently in codon 61 of either the *KRAS* or *NRAS* gene [116].

clinical outlook

ctDNA is thought to represent an average of the whole tumour genome, which may be more accurate than a tissue biopsy or analysis of a small number of CTCs. All liquid biopsy techniques still provide only a snapshot of the current status of the tumour genome, but ctDNA characterization is minimally invasive, cheap, and as such can be repeated on demand. Clinical trials are now urged to facilitate the rapid implementation of this technique into routine clinical use.

conclusions

Patients with mCRC can reach an OS of more than 30 months due to novel treatment approaches including regorafenib and TAS-102. In addition, patients are becoming increasingly fit after the failure of standard treatment options, thus there is a need for novel and individualized therapies in the third-line setting and beyond. Modern pharmacogenomics strategies are allowing reconsideration of known targets, such as *HER2* and *BRAF*, based on precision medicine approaches. The latter include the use of liquid biopsies as a tool to characterize the cancer genome, detect actionable targets at the actual time of treatment initiation, and monitor the dynamic changes occurring under drug selection pressure. Other approaches include targeting novel pathways such as the Wnt signalling pathway and immunotherapies.

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