From tumour heterogeneity to advances in precision treatment of colorectal cancer

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Abstract | In recent years, the high heterogeneity of colorectal cancer (CRC) has become evident. Hence, biomarkers need to be developed that enable the stratification of patients with CRC into different prognostic subgroups and in relation to response to therapies, according to the distinctive tumour biology. Currently, only *RAS*-mutation status is used routinely as a negative predictive marker to avoid treatment with anti-EGFR agents in patients with metastatic CRC, and mismatch-repair status can guide the use of adjuvant chemotherapy in patients with early stage colon cancer. Advances in molecular biology over the past decade have enabled a better understanding of the development of CRC, as well as the more-precise use of innovative targeted therapies for this disease, and include three fundamental achievements. First, the availability of large databases to capture and store the genomic landscape of patients with CRC, providing information on the genes that are frequently deregulated in CRC. Second, the possibility of using gene-expression profiling to differentiate the subtypes of CRC into prognostic groups. Third, results from highly sensitive next-generation sequencing analyses have led to an appreciation of the extensive intratumoural heterogeneity of CRC. Herein, we discuss these advances and place them into the clinical context, and present the novel targets and therapeutic opportunities that are on the horizon.

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The progressive development of colorectal cancer (CRC) provides a model of tumour development[1–4]. CRC is a heterogeneous and molecularly complex disease. Importantly, it has become clear that developments in molecular staging add clinically relevant prognostic and predictive information to the classic staging system, in which patient with CRC can be classified into four different prognostic groups based on the extent of the primary tumour, the involvement of regional lymph nodes, and the presence/absence of distant metastases. The consequences of this complexity for clinical management of CRC are beginning to materialize. Currently, molecular staging has identified patient subgroups that benefit from novel treatments, as well as subgroups that do not benefit from treatments that were previously considered as standard. In this Review, we will discuss the advances our understanding of CRC development, and the current implications of CRC heterogeneity on diagnosis and treatment of the disease.

Colorectal cancer development

CRC is a prime example of how tumours can progress along the disease continuum in a stepwise fashion. Mutations affecting critical genes that regulate cellular proliferation, differentiation, and death accumulate in neoplastic cells, providing them with a survival advantage over the surrounding normal intestinal epithelium[1]. These altered genes cause aberrant expansion of premalignant tissue into adenomas, which have the potential to fully transform into invasive carcinomas that arise as a consequence of additional genetic aberrations[2,3]. The order in which mutations accumulate during the development of CRC is not random[4]. Aberrations in certain genes, such as *APC* and *KRAS*, have been shown to affect early polypoid lesions, and other genetic events are usually observed only when the disease is more advanced, such as *TP53* inactivating mutations[2,4]. This situation, in which specific mutations are associated with particular stages of tumour development, correlates with specific histopathological disease stages; this disease continuum scenario has been a central tenet in CRC research for many years.

Research findings suggest additional molecular complexity that has enhanced our understanding of the biology of CRC and its clinical management. Next-generation sequencing (NGS) studies of the entire CRC genome have revealed that the number of mutations in these cancers is very high — each tumour harbours around

Key points

- Colorectal cancer is a heterogeneous disease, at the intertumoural and intratumoural level, with molecularly-defined subgroups that differ in their prognosis and response to treatment
- Currently, only DNA mismatch-repair status, *RAS*-mutation and possibly *BRAF*-mutation status influence clinical decision-making, although the number of prognostic/predictive biomarkers is increasing
- A transcriptome-based classification of CRC into four consensus molecular subtypes, which differ in their biology and prognosis, and probably also in their responsiveness to treatment, has been reported
- International collaborations and innovative study designs are warranted to drive progress in the clinical development of subgroup-specific treatments

75 mutations[5,6]. Furthermore, individual CRCs contains no less than ~15 mutations that are predicted to be drivers of the disease. The extensive heterogeneity detected between cancers is remarkable, with very few mutations being shared between two given primary CRCs, even when so-called 'driver genes' have a pivotal role in the development of the disease[5,6]. These findings highlight that CRC is genetically very heterogeneous and indicates that therapeutic interventions targeted at specific molecular aberrations are likely to be effective in only a small proportion of patients. Additional research into the development of CRC has established that multiple different histopathological sequences might be involved. The traditional adenoma–carcinoma sequence is thought to be responsible for only a proportion (~50–60%) of CRCs; alternative disease-development routes, such as the serrated pathway characterized by serrated adenomatous lesions that frequently display *BRAF* mutations[7], and colitis-associated CRC development with *TP53* mutations[8,9], are thought to account for the other CRC cancers. Understanding the various developmental trajectories of CRC is critical because the different pathways directly affect the clinical course of the disease. For example, CRCs that display gene-expression profiles closely matching serrated precursor lesions have a poor prognosis and display different response to therapies compared to CRCs associated with the adenoma–carcinoma sequence[10]. Furthermore, tumours can develop via a microsatellite instability (MSI)/CpG-island-methylator phenotype (CIMP)-route, and these tumours are often located in the right colon and have a favourable outcome when detected before disease dissemination[11]. These insights highlight that, after detection of CRC precursor lesions at colonoscopy, intervention and follow-up monitoring needs to be tailored to the specific lesion detected, which is currently an active area of research.

Current standards of care for CRC

Major improvements in outcome for patients with early stage CRC have been achieved with the use of adjuvant chemotherapy[12], which increases the cure rate in patients with stage III colon cancer, and as a result of improvements in surgical technique and neoadjuvant (chemo)radiotherapy, which improve the rate of local tumour control in those with early stage rectal cancer[13,14]. The prognosis of patients with distant metastases has been markedly improved by the availability of new and effective cytotoxic and targeted agents, as well as the more-frequent use of surgical resection of metastases. Further improvements are expected owing to the ongoing implementation of screening programmes for CRC with faecal occult blood testing. Colon and rectal cancer are associated with distinct molecular properties[15] and differ in their response to adjuvant chemotherapy[16], of which the benefit is much more clearly established in colon than in rectal cancer. However, the different anatomical location of these tumours is the aspect that predominantly necessitates a tailored therapeutic approach: rectal cancer involves more-complex surgery, and neoadjuvant (chemo)radiotherapy is used depending on the clinical stage according to MRI. Current data do not indicate a clinically relevant difference for the treatment of distant metastases between colon and rectal cancer.

Early stage disease

Surgery is the mainstay treatment in patients with early stage disease, which is defined as cancers that have only invaded locally (stage I–II), or that present with regional lymph-node metastases (stage III). As expected, the relapse rates following surgery increase with more advanced disease stage or for tumours with unfavourable characteristics. Adjuvant chemotherapy provides a survival benefit in patients with stage III disease, and possibly in those with high-risk stage II colon cancer[12]. High-risk stage II CRC is currently defined by clinical characteristics, which include T4 stage, a low number (<10–12) of regional lymph nodes examined, poorly differentiated tumours, presence of extramural vascular invasion, and/or presentation with obstruction or perforation. The potential benefit from adjuvant chemotherapy is currently predicated on the relatively high incidence of recurrence in these groups, and not on a distinct biological sensitivity to therapy. However, the long-term follow-up data of the pivotal MOSAIC trial call into question the benefit of adjuvant chemotherapy in patients with high-risk stage II colon cancer[12]. DNA mismatch repair (MMR) deficiency status is the only biomarker that can be used to select patients with high-risk stage II colon cancer for adjuvant chemotherapy[17]. The addition of targeted drugs to standard adjuvant chemotherapy seems to be ineffective[18,19]. Mechanisms that have been proposed to explain the failure to improve outcomes in patients with microscopic residual disease are the absence of tumour neoangiogenesis for the anti-VEGF antibody bevacizumab, and an epithelial-to-mesenchymal transition (EMT) phenotype for the anti-EGFR antibody cetuximab. Moreover, the benefits of adjuvant chemotherapy are limited; many patients have disease relapse despite therapy, whereas some patients never have a relapse despite no treatment. Most of the clinical data on which the selection of patients for adjuvant chemotherapy is based were published over 10 years ago. In the past decade, diagnostic tools, pathological analysis of tumour samples, and the quality of surgery have improved considerably. These improvements implies that many patients might be overtreated with adjuvant chemotherapy[20]. Indeed, reassessment

of the current criteria and better predictive biomarkers for the selection of patients who might benefit from adjuvant chemotherapy are urgently needed.

Metastatic disease

In patients with metastatic CRC, surgical resection of metastases, either upfront or after downsizing by systemic induction regimens, offers the best chance for cure; however, this option in only available for a minority of patients because most patients present with more-advanced and, therefore, unresectable metastases. The optimal induction regimen for systemic therapy has not been established and is currently being investigated in a prospective trial (CAIRO5)[21]. In this trial, patients with liver metastases that are unresectable according to predefined criteria and have *RAS*/*BRAF*-wild-type tumours are being randomly assigned to receive doublet chemotherapy plus either bevacizumab or panitumumab; whereas patients with unresectable liver metastases and tumours harbouring *RAS*/*BRAF* mutations are being assigned to receive bevacizumab with either doublet or triplet chemotherapy. Resectability status is monitored by a panel of liver surgeons and radiologists. If resection is not a realistic goal, systemic treatment (with chemotherapy and targeted therapy) substantially prolongs overall survival[22–36]. Active chemotherapeutic agents include the fluoropyrimidines (5-fluorouracil (5-FU) and capecitabine), irinotecan, oxaliplatin, and trifluridine/tipiracil[22,23]. Chemotherapy can be administered in combination or sequentially, depending on the characteristics of the patient[24,25]. The benefit of chemotherapy is further increased by the addition of targeted drugs, such as bevacizumab, and in patients with *RAS*-wild-type tumours, by the addition of cetuximab or panitumumab[26–31]. These anti-EGFR antibodies have efficacy as monotherapy in previously treated patients[32,33]. In the past few years, other targeted drugs have demonstrated a benefit over standard care, such as aflibercept (a decoy receptor for VEGF-A, VEGF-B and PIGF) and ramucirumab (an antibody against VEGFR-2), both in combination with chemotherapy in the second-line setting, and regorafenib (a multikinase inhibitor) as monotherapy in the refractory setting[34–36]. The current standard first-line treatment in patients with *RAS*-mutated tumours consists of chemotherapy (with single agent, doublet, or triplet regimens) plus bevacizumab[26–29]. In patients with *RAS*-wild-type tumours, the optimal sequence of anti-VEGF and anti-EGFR antibodies remains a matter of debate[37–39]. The choice of a systemic treatment strategy should depend on tumour-related and disease-related characteristics (extent of disease, symptoms, biomarkers), patient-related factors (comorbidity, socioeconomic factors, expectations of patients), and treatment-related factors, such as toxicity, with the intention to optimally expose patients to the available effective drugs during the course of their disease (continuum of care)[40,41] Lastly, continuous rather than intermittent inhibition of growth signalling is considered the preferred strategy, as shown in two randomized trials that demonstrated a better outcome for maintenance treatment with bevacizumab in combination with fluoropyrimidine monochemotherapy until disease progression compared with observation, and in one of these trials, also with bevacizumab monotherapy[42,43]. Fluoropyrimidine monochemotherapy alone has not been formally tested as a control, but the added value of bevacizumab to fluoropyrimidine monotherapy in metastatic CRC argues in favour of use of the combination[44].

With limited exceptions, all systemic treatments are administered as a 'one-size-fits-all' approach, with only a subset of patients experiencing a benefit. Thus, predictive biomarkers are urgently needed in the metastatic setting. These tools should enable patients and oncologists to make more-informed treatment decisions, both for chemotherapeutic approaches and novel targeted strategies, in order to optimize efficacy and patient well-being, and to reduce the costs associated with patients not deriving benefit from treatments.

Relevance of intertumour heterogeneity

Intertumour heterogeneity refers to the observation that CRCs in distinct patient subgroups present with vastly different genetic make-ups, histopathological features and clinical behaviours. Similarly, intratumoural heterogeneity relates to the genetic heterogeneity between cancer cells within a single tumour. These differences can be related to genetically distinct populations (clones) present in the cancer, or owing to various degrees of cellular differentiation. Herein, we discuss the clinical relevance of various types of tumour heterogeneity.

Genetic heterogeneity of early disease

Several studies investigating how anatomic site affects tumour progression have shown a worse prognosis for right-sided versus left-sided colon cancers[45–49]. Differences between right-sided and left-sided colon cancers have been postulated to arise in response to complex genetic and epigenetic changes caused by inherited and environmental factors, which do not abruptly change at the splenic flexure[49].

The single most-informative genetic characteristic in early stage colon cancer is undoubtedly MSI[50]. MSI tumours have an impaired MMR system, and consequently accumulate a very high level of mutations. MSI is caused by a mutation in one of the MMR genes or by hypermethylation of the *MHL1* promoter[2]. The majority of these tumours can be detected by gene-expression profiling, which reveals how this feature is associated with a radically different biology to other CRCs[5,10,51,52]. The recognition of MSI is of major clinical relevance for several reasons. First, MSI tumours frequently occur in the context of Lynch syndrome, an inheritable condition (caused by germline mutations in one of the MMR-related genes) that is associated with increased colon cancer risk[2]. Identification of patients with MSI is important to enable adequate counselling to be provided, and can allow affected family members to be identified. Second, patients with early stage MSI tumours have a better prognosis than those habouring microsatellite stable (MSS) tumours[53]. Third, the recurrence rate in patients with stage II MSI tumours is too low to justify adjuvant chemotherapy for all patients. Stage II and III

patients with MSI colon cancer should not be treated with fluoropyrimidine monotherapy, because this strategy has been shown to be ineffective, and impaired disease outcome has been noted in patients with stage II disease[17]. Patients with stage III MSI tumours do benefit from oxaliplatin-containing adjuvant therapy, which has been confirmed in a subgroup analysis of the MOSAIC study[12]. Fourth, a potential benefit of bevacizumab as adjuvant treatment in combination with chemotherapy in early stage colon cancer has been suggested in patients with MSI tumours, and might be explained by a bevacizumab-induced disruption of the immunosuppressive microenvironment in these immunogenic tumours[54]. Preliminary results of the adjuvant QUASAR2 study have not confirmed this observation[55], for which no explanation is currently available. Lastly, the results of Sinicrope *et al.*[53] suggest a difference in response to adjuvant treatment for germline versus sporadic MSI cancers. The findings showed a benefit in disease-free survival for 5-FU treatment versus observation or treatment lacking 5-FU in patients with suspected germline mutations in MMR-related genes, but not in those with sporadic MMR-deficient (dMMR) tumours[53]. This finding might be explained by the frequent presence of *BRAF* mutations in sporadic dMMR tumours showing hypermethylation of multiple genes (that is, CIMP tumours)[56]. *BRAF* mutations are absent in germline dMMR colon cancers[53,57], an observation that illustrates the complexity of the prognostic and potential predictive value of MMR status and *BRAF*-mutation status (TABLE 1). In the future, *BRAF*-mutation status in combination with MSI status might help to better-select patients for adjuvant treatment[58–60]. This observation, however, requires more in-depth analysis and confirmation. Thus, at present, the only biomarker with predictive value for adjuvant treatment used in clinical practice is MSI/dMMR: patients with MSI high-risk stage II tumours should not be treated with adjuvant chemotherapy, and patients with MSI stage III tumours should be treated with oxaliplatin-based adjuvant chemotherapy only, and not with fluoropyrimidine monotherapy. Patients with *KRAS*-mutant MSS stage III colon cancer have a poor prognosis[61], and have a different dissemination pattern often associated with frequent lung metastasis[62,63]. These findings support the use of this mutation to stratify patients in future clinical trials; however, information on the clinical implications for stage I–III cancers is lacking. The absence of CDX2 expression has shown promise as a predictive marker of response to adjuvant chemotherapy in high-risk stage II colon cancer[64]; however, the predictive power in patients with CDX2-negative high-risk stage II colon cancers was low, and the results require further validation. Other common mutations in early stage cancers, such as *SMAD4*, *TP53* and *APC*, only display a very weak association with disease outcome in CRC[50].

Transcriptomic heterogeneity in early stage disease

Genetic aberrations contribute to tumour heterogeneity, but the clinical manifestation of cancers and the underlying tumour biology is shaped by many additional tumour characteristics. These include the epigenetic aberrations, the composition of the stroma and how this relates to the local immune response, and the extent of vascularization and hypoxia[65]. All these aspects are integrated in the tumour transcriptome. Different approaches have been taken to use gene-expression data to stratify patients[66]. Traditionally, supervised analyses have been performed to identify gene signatures that are associated with poor disease outcome. First, gene-expression data generated from early stage colon cancers are used to identify a subset of genes, expression of which is associated with a poor disease outcome. Next, a signature comprising these gene products is assembled and validated in an additional dataset. Several commercial assays have been developed that facilitate the use of these profiles in the clinic. Examples include the Oncotype DX 12-gene RT-PCR assay (Genomic Health, USA)[67,68] and the ColoPrint 18-gene microarray-based classifier (Agendia Inc., USA)[69]. ColoPrint has been shown be of greater prognostic value in patients with stage II colon cancer compared with that of traditional clinicopathological assessment of high-risk features (such as T4 tumours, poorly differentiated morphology, and others); in multivariate analysis, this assay was predictive of disease-free survival only in a cohort of 135 patients with stage II colon cancer[70]. A discordance rate of 48% was reported for the risk of disease recurrence when ColoPrint and standard clinical criteria were compared[69]. Consequently, this assay might be used in the future to guide adjuvant therapy decisions in this population, although a potential limitation to the use of this tool in the clinic is the need for fresh-frozen tumour material. Moreover, further evidence is needed, to identified whether patients at high-risk actually benefit from currently used adjuvant therapies. In this respect, data from the NSABP C-07 study[71], in which patients with stage II/III CRC were randomly assigned to receive either adjuvant 5-FU or

Table 1 | **Prognostic and predictive value of DNA-mismatch repair and *BRAF*-mutation status**

Biomarker present	Stage II		Stage III		Stage IV/metastatic disease	
	Predictive*	Prognostic	Predictive*	Prognostic	Predictive*	Prognostic
MSI/BRAF^{mut}	Yes	Favourable	Yes	Favourable	Unknown	Unfavourable
MSI/BRAF^{wt}	Yes	Favourable	Yes	Favourable	Yes	Unfavourable
MSS/BRAF^{mut}	No	Unfavourable	No	Unfavourable	Yes	Unfavourable
‡MSS/BRAF^{wt}	NA	NA	NA	NA	NA	NA

MSI, microsatellite instability; MSS, microsatellite stable; mut, mutated; NA, not applicable; WT, wild-type. *Treatments that relate to predictive value are explained in the text. ‡MSS/BRAF^{wt} serves as the reference group.

Table 2 | **Transcriptional identified consensus molecular subtypes (CMS)**

Tumour subtype	CMS1 MSI/immune	CMS2 canonical	CMS3 metabolic	CMS4 mesenchymal
Proportion*	~15%	~40%	~10%	~25%
Genomic features	Hypermutated	SCNA high	Mixed MSI	SCNA high
Genetic drivers	*BRAF*	*APC*	*KRAS*	Unknown
Associated precursors	Serrated	Tubular	Unknown	Serrated
Gene-expression signature	Immune	Wnt/MYC activity	Metabolic deregulation	• TGFβ / EMT • High stromal content
Prognosis	Intermediate	Good	Intermediate	Poor

EMT, epithelial–mesenchymal transition; MSI, microsatellite instability; SCNA, somatic copy-number alterations.*Approximately 10% of cases are not reliably classified into one tumour subtype. Adapted with permission from Guinney J. *et al.* The consensus molecular subtypes of colorectal cancer. *Nat. Med.* **21**, 1350–1356 (2015).

5-FU plus oxaliplatin therapy, have provided a first clue. Retrospective classification of patients enrolled in this trial using the Oncotype DX tool demonstrated a similar benefit of oxaliplatin-based therapy for different risk categories, suggesting an increased absolute benefit of this agent in patients identified as high-risk by Oncotype DX–based stratification[72]. However, these data do not exclude a possible benefit from adjuvant treatment for patients identified as low-risk. The predictive value of these tools needs further investigation, and a prospective study using paraffin-embedded tissue samples and stratification using ColoPrint is currently ongoing[73].

Notwithstanding the potential clinical utility of these gene-expression arrays for detecting patients at high risk of recurrence, this approach provides little biological insight into the disease. Moreover, this approach does not enable the identification of novel and rational targets for therapy in patient subgroups. To circumvent these shortcomings, several groups have used a radically different strategy to identify molecular CRC subtypes using an unbiased approach — that is, independent of clinical features of the disease[10,74–79]. These studies have resulted in a series of classifications that, for example, can detect a canonical colon cancer with an epithelial expression profile and a relatively good prognosis, a mesenchymal colon cancer subtype associated with a poor disease outcome, and a subtype that is strongly associated with MSI cancers and a favourable disease outcome[80]. Intriguingly, none of these subtypes can be recognized based on a specific genetic event, signifying that the genetic background of a cancer is only partially responsible for its gene-expression profile and clinical behaviour, and that the developmental route to progression and the tumour microenvironment are equally critical. An integration of these transcriptome-based disease classifications has now enabled the definition of four consensus molecular subtypes (CMS1–4)[81] (TABLE 2). CMS1 represents a subgroup of cancers with a good prognosis and a strong association with MSI tumours. CMS2 comprises cancers with an epithelial-cell-like gene-expression profile and a high degree of chromosomal instability. CMS3 cancers display marked metabolic deregulation, while CMS4 cancers display mesenchymal features, extensive stromal invasion and hold a poor prognosis. Given the extensive biological differences between these subtypes, responsiveness to therapies is also likely to differ for each subtype. Indeed, metastatic tumours of the mesenchymal subtype display resistance to anti-EGFR monotherapy independent of *RAS*-mutation status[10,82]. Similar evidence indicates that patients with mesenchymal colon cancers (stage II/III) do not benefit from adjuvant chemotherapy[79]. Of note, these insights are all derived from retrospective analyses, with associated shortcomings, and thus dedicated prospective studies are needed to establish the relevance of the CMS for guiding treatment decisions. We advocate the use of the CMS in the prospective evaluation of novel treatment modalities in order to increase the likelihood of identifying novel active compounds and to ensure that new treatments can be readily introduced in patient groups that will benefit most.

Metastatic disease and heterogeneity

Prognostic implications and biomarkers. Similar to early stage disease, both clinical and molecular data have shown that patients with metastatic CRC have a heterogeneous prognosis and response to treatment. Few predictive biomarkers are available, resulting in the use of a 'one-size-fits-all' approach, whereby many patients are unnecessarily exposed to the toxic effects of (often very expensive) treatments. In addition to 'classic' clinical prognostic factors, such as performance status, extent of disease, and serum LDH levels, BMI has been shown to have prognostic value[83]; if confirmed, further research is warranted to explain the biological mechanism behind the relationship between BMI and prognosis. The resection status (yes versus no) of the primary tumour has also been identified as a potential prognostic factor in patients with synchronous metastases[84], which is currently being assessed in prospective clinical trials[85–87]. In addition to known predictive value for the efficacy of treatment with anti-EGFR antibodies, *KRAS*-mutation status might also have prognostic value[88]. Data indicate that anatomical site (proximal versus distal from the splenic flexure) might be another important prognostic parameter, independent of mucinous histology and *BRAF*-mutation status[48], but further research is needed to clarify this relationship.

Influence on response to chemotherapy. In general, systemic chemotherapy is the treatment modality that provides the greatest benefit to patients with metastatic disease. Despite intensive research on predictive biomarkers of responsiveness to chemotherapy, no clinically useful markers have been identified[89]. Similarly, currently no predictive markers are available to guide bevacizumab therapy[90]. In the ongoing MAVERICC trial[91], previously untreated patients with mCRC are being randomly assigned to receive either FOLFOX6 (a regimen comprising 5-FU, folinic acid, and oxaliplatin), or FOLFIRI (5-FU, folinic acid, and irinotecan); bevacizumab is being added to each treatment arm and serum VEGF-A levels are being determined. The results of these analyses of

the predictive value of VEGF-A levels and the efficacy of bevacizumab-containing therapies are eagerly anticipated[91]. Furthermore, in the MAVERICC trial[91], the expression of the excision repair cross-complementation group 1 (*ERCC1*) gene is being investigated as a potential predictive marker of resistance to platinum compounds; however, in a preliminary analysis, no association between *ERCC1* expression levels and the efficacy of oxaliplatin could be detected[91] — in contrast to findings from earlier studies[92,93]. Other therapeutic biomarkers are currently under development, and range from immunohistochemical assays to high-end genomic approaches. For example, detection of mutations in circulating DNA can predict efficacy to regorafenib[94], although no specific genetic variant was associated with drug activity.

Implications for anti-EGFR therapy. As we have alluded to, *KRAS*-mutation status is the strongest predictive biomarker in the management of CRC. Initially, patients with tumours harbouring *KRAS* exon 2 mutations were show to lack responsiveness to anti-EGFR therapy[95,96]. Subsequently, additional *KRAS* and *NRAS* mutations (commonly summarized as *RAS* mutations) have also been found to be of predictive value, with detrimental clinical effects in patients with *RAS* (*KRAS* and/or *NRAS*)-mutant tumours upon anti-EGFR treatment[97]. Thus, anti-EGFR treatment is currently only indicated in patients with *RAS*-wild-type tumours.

As BRAF is downstream of RAS in the MAPK/ERK signalling axis, *BRAF*-mutated cancers would be expected to display a similar degree of resistance to anti-EGFR therapy as *RAS*-mutated cancers. Of note, patients with metastatic CRC harbouring *BRAF* mutations have an extremely poor prognosis[98]. The low prevalence of *BRAF* mutations (<9%), which are almost mutually exclusive with *RAS* mutations[98], hampers the feasibility of prospective randomized trials in this subgroup, but trials have been initiated. The predictive value of *BRAF*-mutation status for anti-EGFR treatment is also difficult to assess (owing to the low prevalence of *BRAF* mutations) in patients with metastatic CRC[99,100]. Nevertheless, meta-analyses have shown limited or no clinical benefit of anti-EGFR treatment in this subset, which argues against the use of this treatment in patients with *BRAF*-mutated tumours[99,100]. To date, the best results in patients with a *BRAF*-mutant CRC with have been achieved with triplet chemotherapy (5-FU, folinic acid, oxaliplatin, and irinotecan; FOLFOXIRI) plus bevacizumab[88], which supports the strategy of exposing this group to all available drugs with efficacy for as long as possible during their course of disease — many of these patients are not able to receive salvage treatments owing to their poor prognosis.

Additional molecular features associated with resistance to anti-EGFR therapy in many preclinical studies, include *PIK3CA* and secondary *EGFR* mutations, but these alterations are not assessed in routine screening before therapy[101–104]. Intriguingly, many of these resistance-conveying aberrations converge on the same few pathways, which might enable therapeutic targeting[105]. *HER2* amplification has also been identified as a driver of resistance to anti-EGFR therapy[106]. Preliminary clinical data have revealed that *HER2* is amplified in around 5% of patients with *KRAS*-wild-type metastatic CRC, and that these patients might benefit from dual HER2 inhibition with trastuzumab and lapatinib[107].

RAS-mutation status can only be used as a negative predictive marker (that is, *RAS* mutation is predictive of a lack of responsiveness, but a *RAS*-wild-type status does not guarantee a response) and, indeed, only a subset of patients with *RAS*-wild-type tumours benefit from anti-EGFR treatment; therefore, further research is warranted to better stratify patients for therapy, and thereby reduce costs and adverse events associated with suboptimal therapy. A promising avenue of further research is the molecular CRC subtypes identified by gene-expression profiling, which display radically different responses to anti-EGFR therapy independent of *RAS*-mutation status[10]. Furthermore, the value of clinically relevant mutations could be improved by analysing circulating plasma DNA rather than archival tumour tissue[108], which might help eludicate acquired resistance mechanisms[109].

BRAF-targeted therapy. BRAF inhibitors that display very high efficacy in melanomas harbouring a $BRAF^{V600E}$ mutation are ineffective as monotherapy in *BRAF*-mutated mCRC[110,111]. Inhibition of BRAF in CRC cells results in rapid activation of the EGFR/PI3K pathway via a feedback activation loop that is absent in melanoma cells[112,113]. Perhaps melanocytes and colonic epithelial cells originate from different germ lines and present with radically differently signal transduction networks. Preclinical studies have demonstrated promising results with the use of combination therapy with BRAF inhibitors, EGFR and/or PI3K inhibitors[112], and preliminary results of clinical trials have shown objective responses to such treatment combinations, but in only a minority of patients (response rates of 12–32%)[114–118]. Lastly, a gene-expression signature that is derived from *BRAF*-mutant CRCs is also detected in 20% of tumours that lack *BRAF* mutations. Preclinical data indicate these so-called *BRAF*-like tumours have selective sensitivity to vinorelbine *in vitro* and *in vivo*[119].

Immunotherapy. Promising results have been reported with the use of pembrolizumab, an antibody to programmed cell death protein 1 (PD-1) in previously treated patients with metastatic CRC and dMMR tumours, with an objective response and disease control (objective response or stable disease for ≥12 weeks) in 4 and 9 of 10 patients, respectively; objective responses were seen in 5 of 7 patients with metastatic non-CRC dMMR tumours. In 18 patients with metastatic CRC and MMR-proficient tumours, an objective response and disease control were observed in 0 and 2 patients, respectively[120]. The use of this antibody was linked to a biomarker (MSI, and potentially CMS1), on the basis that dMMR tumours could be sensitive to immunotherapy owing to the high somatic mutational load. Moreover, dMMR cancers contain prominent lymphocyte infiltrates, a finding consistent with an immune

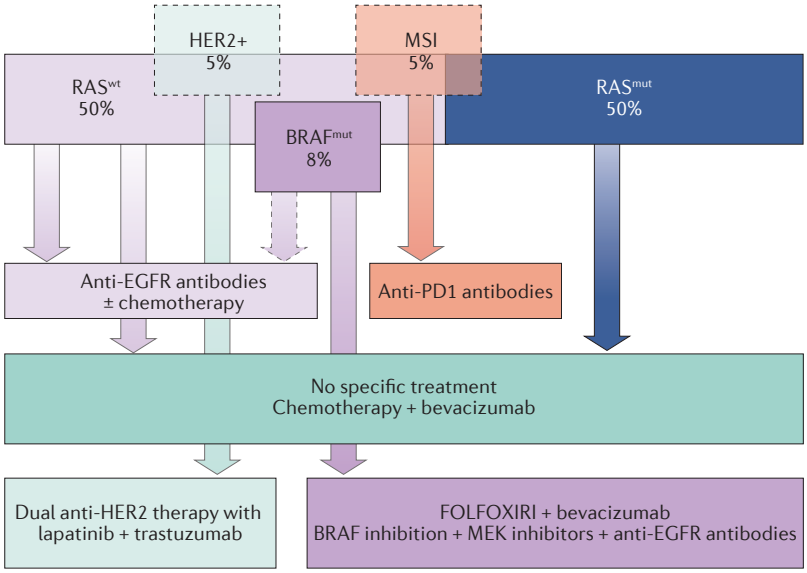

Figure 1| **Proposed landscape of molecularly targeted treatments for metastatic colorectal cancer.** The schematic summarizes the biomarker-based treatment options available and the typical proportions of patients in each biomarker subgroup. FOLFOXIRI, 5-fluorouracil, folinic acid, oxaliplatin, and irinotecan; MSI, microsatellite instability; mut, mutant, PD-1, programmed cell-death protein 1; wt, wild type.

response. Interestingly, none of the tumours from the 10 patients with dMMR[120] harboured *BRAF* mutations; thus, the efficacy of anti-PD1 treatment in patients with dMMR and a *BRAF*-mutated tumour is unknown. dMMR is rare in metastatic CRC[121] and, in contrast to MSI in early stage disease, defines a group of patients (possibly driven by *BRAF*-mutation status) with a less favourable prognosis[122]. Further research should help to resolve whether immune-checkpoint inhibitors might be beneficial as adjuvant treatments for patients with early stage disease, in which MSI is more-common, or whether these agents are active in selected patients lacking MSI, but who might nonetheless present with T-cell immune infiltrates. A higher neoantigen mutational load was positively correlated with T-cell lymphocytic infiltration and cancer-specific survival in patients with MSI and MSS CRC tumours[123], which might enable selection of the patients most likely to benefit from experimental immunotherapies.

Identification of novel therapies. Novel emerging targets are proteins from the Wnt pathway, which is activated in virtually all CRCs, and that can be targeted, for example, with tankyrase inhibitors[124,125]. Tankyrase inhibitors prevent poly(ADP-ribosylation)-dependent degradation of axin, resulting in β-catenin destabilization and impairment of Wnt signalling activity, thereby reducing cell proliferation, and inducing cell differentiation and/or death[124,125]. Importantly, these agents exert Wnt-inhibitory properties in the presence of *APC* mutations — a downstream component of the pathway: tankyrase inhibition not only reduced the growth of *APC*-mutant CRC tissue in xenograft models[124], but also halted tumour development in mice lacking *Apc*[125]. Interestingly, this tumour growth reduction was accompanied by enhanced cell differentiation and reduced clonogenicity, corroborating the importance of the Wnt pathway in CRC stem cells[124]. Furthermore, combining tankyrase inhibitors with targeted agents, such as AKT and PI3K inhibitors, or chemotherapy was effective in preclinical models of colon cancer[126]. Wnt signalling is impaired by inhibitors that target porcupine, a protein that prevents secretion of Wnt proteins by inhibiting their palmitoylation, which is required for membrane shedding of these signalling molecules[127,128]. For example, the compound LGK947, a potent and specific small-molecule inhibitor of porcupine, is in phase I testing in patients with Wnt-driven cancers[129]. Owing to its inhibition of Wnt-protein secretion, porcupine inhibition is expected to be highly effective in Wnt-driven cancers that do not harbour downstream Wnt pathway-activating mutations in *APC* or *CTNNB1*, but instead rely on upstream activating events[130]. These include the reported rare fusion events involving the *RSPO2* and *RSPO3* genes, encoding R-spondin proteins that positively regulate Wnt signalling, that have been detected in *APC*-wild-type CRCs[131,132]. The porcupine inhibitor ETC-159 has demonstrated clear efficacy in *RSPO*-translocation-bearing xenografts derived from patients with CRC[130]. Similarly, inhibition of Wnt-secretion using the the small-molecule porcupine inhibitor, Wnt-C59, has demonstrated activity against mouse *Rnf43* and *Znrf3* double-mutant intestinal tumours (these genes encode negative regulators of Wnt signalling)[133]. Finally, targeting R-spondin-3 in *PTPRK–RSPO3*-fusion-positive human CRC tumour xenografts inhibits tumour growth and promotes differentiation, providing a viable therapeutic option for this rare subtype[134].

The MAPK signalling cascade that is invariably activated, for example, via *RAS* and *BRAF* mutations, is another emerging target for CRC therapy. This pathway could potentially be targeted downstream of BRAF with the use of MEK inhibitors. Clinical data are available from early phase studies of these agents. Combined inhibition of BRAF and MEK using dabrafenib and trametinib in *BRAF*-mutant mCRC resulted in inhibition of MAPK signalling in all patients, but clinical efficacy was only demonstrated in a subset of patients[114]. The patient subgroup most likely to benefit from this approach remains to be identified; mutations that were proposed to convey resistance, including *PIK3CA*, were not predictive of responsiveness to therapy in the metastatic setting. Furthermore, less-promising results were obtained in patients with *RAS*-mutant CRC tumours, suggesting that MEK inhibitors might be beneficial only for patients with *BRAF*-mutant cancers[135]. This finding correlates with earlier data that single-agent MEK inhibition with RO4987655 was effective in some patients with *RAS* and *RAF*-mutant NSCLC and melanoma, but not in those with *RAS*-mutated CRCs[136]. Taken together, these data indicate that MEK inhibitors might be most promising as BRAF-inhibition-potentiating agents in patients with *BRAF*-mutant cancers[135,136]. Indeed, trials combining BRAF inhibitors with MEK inhibitors and anti-EGFR agents or PI3K inhibitors are currently underway[118] (FIG. 1).

Every improvement in the detection of predictive markers for current and innovative drugs will help to identify smaller subgroups in which large-cohort prospective randomized phase III trials will be challenging to perform. To solve this problem, worldwide collaboration and innovative research approaches are urgently needed; for example, observational studies providing a dynamic infrastructure for conducting prognostic, predictive, biological, interventional, and cost-effectiveness studies, including multiple cohort randomized trial designs[137,138].

Relevance of intratumour heterogeneity

Awareness of intertumour heterogeneity has existed for a long time; however, the extent of intratumour heterogeneity has only been recognized in the past decade. The challenges associated with intratumour heterogeneity are immense and include minimal residual disease and the emergence of therapy resistance. Herein, we outline important concepts related to intratumour heterogeneity and discuss novel therapeutic paradigms.

Intratumoural heterogeneity relates to genetic heterogeneity, functional heterogeneity, and nongenetic (such as epigenetic) heterogeneity. Genetic intratumour heterogeneity is a consequence of evolutionary processes associated with cancer development and progression. During the oncogenesis process, genetic aberrations accumulate continuously, and provide the cell with an enhanced ability to expand, which increases the mutation prominence of the tumour population. The result of this ongoing process is that cancers are genetically heterogeneous, with numbers of coexisting clones that vary over time depending, among others factors, on the mutation rate and selective pressures[139]. These clones have distinct functional properties, such as the ability to form metastases or respond to specific therapies.

Heterogeneity also exists between genetically identical cancer cells. The most-critical distinction is between fully differentiated, non-clonogenic cancer cells that have lost the ability to contribute to tumour growth, and immature stem-cell-like cells with extensive self-renewal potential, also known as cancer stem cells, which are believed to fuel long-term cancer growth and metastasis[137]. Furthermore, cancer stem cells are reportedly resistant to conventional chemotherapeutic agents and are, therefore, believed to be the seeds of disease relapse[140].

Genetic intratumour heterogeneity

In the past few years, large-scale studies have defined the genetic intratumour heterogeneity of various malignancies, including CRC. For example, the use of TCGA data from nine different cancer types has facilitated establishing that driver events in genes (such as *KRAS*) are more likely to be present in virtually all cancer cells compared with non-driver events, suggesting that these mutations occur early in tumour development[141]. Data from a more-detailed analysis of CRC development using intratumour heterogeneity provide insight into which mutations occur at what time in the disease trajectory[142]. These analyses have resulted in the 'big-bang' concept postulating that most driver events in CRC (including *APC*, *KRAS*, and *TP53* aberrations, as well as most subclonal mutations) occur before or early after the transition to advanced carcinoma[142]. Subsequent mutations that accumulate are functionally neutral and, as a consequence, 'clonal sweeps' in established CRCs are extremely rare during normal, unperturbed tumour progression[142].

The subclonal landscape of CRCs has been shown to have direct consequences for therapy efficacy. In one study[143], material from the CAPRI-GOIM trial[144] of first-line cetuximab plus FOLFIRI in patients with *KRAS*-wild-type metastatic CRC was analysed using NGS to determine mutant allele frequencies and estimate clonal prevalences. In this cohort, *KRAS* and *NRAS* mutations were actually found to be present in the vast majority of tumour cells (clonal), whereas *BRAF* and *PIK3CA* mutations were often present in only a subset of cancer cells (subclonal)[143]. These data correlate well with those of earlier studies showing that the use of conventional PCR methods for the analysis of *KRAS*-mutation status is prone to underestimation of the presence of mutations in this gene[145]. Intriguingly, no direct relationship was noted between the proportion of cells with *KRAS* mutations and cetuximab efficacy, with the data suggesting that even tumours with only a minority of cancer cells harbouring *KRAS* mutations display resistance to anti-EGFR agents[143]. In the CRYSTAL trial, however, a relationship was reported between the fraction of *RAS*-mutated tumour cells and the response to anti-EGFR therapy[95], with a benefit for cetuximab combined with chemotherapy reported in patients with tumours harbouring a low prevalence of *RAS* mutations (0.1–5%); a similar threshold (1%) was reported in a separate patient cohort[146]. The presence of subclonal *KRAS* mutations is associated with a reduced response to anti-EGFR agents because the subclones habouring these mutations can act as a reservoir of resistant cells that expand following selective therapeutic pressure to repopulate the tumour. This finding has been confirmed in patients with CRCs who had a relapse after anti-EGFR therapy; analysis of pretreatment and post-treatment samples revealed that *KRAS* mutations became detectable in the circulation before radiological evidence of relapse[109,147]. Other mutations associated with resistance were detected in patients who had a relapse following cetuximab treatment, including *EGFR* aberrations that have been shown to prevent binding of the drug to the extracellular binding domain of EGFR[104]. Cells with these *EGFR* mutations are likely to be present at very low levels in the tumour-cell population and only emerge after cetuximab therapy[104]. Resistance to other agents probably follows similar principles; however, the mechanisms of resistance remain unclear. Intratumour heterogeneity poses enormous challenges to enable precision therapy, because not only the presence or absence, but also the prevalence of specific genetic aberrations in tumours must be determined in order to predict therapeutic efficacy. The current approach to tackle acquired drug resistance involves ways to circumvent

resistance mechanisms by adding additional inhibitors; however, the dynamic and evolutionary nature of cancer progression and the development of secondary resistance require consideration of alternative strategies[148]. The treatment 'dogma' in oncology is to maximize cell death at the initial stages, but this approach enables the rapid outgrowth of resistant clones leading to relapse[145]. By contrast, the aim of 'adaptive therapy' is to control metastatic disease by enabling treatment-sensitive clones to persist at stable levels that, in turn, keep the levels of treatment-insensitive subclones stable, an approach that can potentially extend survival rates[149,150]. Alternatively, the evolutionary trajectory that results in the development of resistance might be associated with transient exploitable vulnerabilities. This notion, referred to as 'temporal collateral sensitivity', might reveal additional cancer-cell sensitivities that have remained undetected in static screens[151]. Critically, these principles are far from clinical application and await further rigorous preclinical testing.

Nongenetic intratumour heterogeneity

Cancer stem cells from patients with CRC can be identified by the detection of cell-surface expression of CD133 or CD44/CD166, elevated aldehyde dehydrogenase activity, and by a hyperactivation of the Wnt pathway[152–156]. Functionally, cancer stem cells are characterized by the ability to form subcutaneous phenocopies of the original human malignancy in immunocompromised mice[157]. Extensive preclinical evidence indicates that tumour cells displaying stem-cell features are resistant to chemotherapy and targeted agents[140,158,159]. For example, irinotecan treatment of xenograft models of human CRC led to an increase in the numbers of tumorigenic cells expressing both CD166 and CD44 (REF. 160). CRC-stem cells express increased levels of antiapoptotic genes and increased levels of multidrug-transporters on the cell surface, which might explain the differential chemosensitivity of these cells compared with non-stem tumour cells[159,161,162]. Furthermore, CRC stem cells reside in protective niches that render them less sensitive to therapeutic pressure than non-stem cells[163,164]. For example, HGF produced by myofibroblasts can preferentially select the CRC cancer stem-cell population and induce resistance to anti-EGFR agents[156,165]. Intriguingly, the cancer stem-cell phenotype is not static, and can be induced in more-differentiated cells following exposure to specific factors produced by tumour-associated myofibroblasts, which includes HGF, osteopontin, and interleukin 17A (IL-17A)[156,166,167]. Interestingly, IL-17A is predominantly produced by fibroblasts exposed to chemotherapy, suggesting that therapies can promote the cancer stem-cell phenotype via modification of the tumour microenvironment[167]. Interference with these signals combined with conventional therapies is a promising avenue of treatment that requires further study.

Future perspectives

The ultimate promise of personalized treatment is that therapy can be specifically tailored for each individual patient, based on clinical and genomic characteristics, such as physical performance status (as well as patient preference), and the biomolecular properties of the cancer encompassing detailed information on driver mutations, immune-cell composition of the stroma, and epigenetic characteristics. Unfortunately, our ability to predict the clinical efficacy of drugs on the basis of preclinical research, or clinical responses in relation to the tumour mutational characteristics is limited[111]. Large pharmacogenomics studies performed in thousands of cell lines have started to meticulously characterize drug tailoring and response[168,169]. With few exceptions, the large majority of differences in responses to a particular drug are not attributed to individual molecular features. Moreover, the majority of associations between drug activity and genetic features are relatively weak[168,169]. To improve the selection of the best drugs for each patient, comprehensive molecular information on the patients' tumours will be required to complete our understanding of the biology of cancer.

The improved expansion of organoid cultures[170] to assess CRC and the establishment of large libraries of CRC tissue grown in immunocompromised mice (xenopatients)[101,106,171,172], will be very important in this endeavour. Both strategies enable the evaluation of drugs in more-relevant preclinical models compared with cultured cell lines supplemented with serum. Efforts are underway to explore if these technologies can be used to screen for drug efficacy in various clinical settings. For example, biopsy samples of CRC material can be expanded either in organoid cultures or in xenograft models, and a 'drug library' screen can then be used to test which agents are effective for each particular cancer[173]. Important outstanding issues that need to be addressed include a fast turnaround time for the use of new model systems and genomic assays, as well as a better understanding of the influence of intratumour variation and how this relates to the sensitivity of drug testing using these methods.

Conclusions

To summarize, molecular testing has greatly contributed to our knowledge of CRC development. Furthermore, it has become clear that CRC is a heterogeneous disease, and molecular subtyping substantially impacts on prognostication, as well as on the selection of treatments for specific stages of disease. Thus, future trials in molecularly unselected patients will probably not provide clinically relevant data. This implies that future clinical trials in CRC should either be restricted to the molecular subtype(s) of interest, or at least should stratify for validated prognostic and/or predictive biomarkers. Novel bioinformatic strategies need to be developed to improve the prediction of responses to therapy on the basis of molecular data, and will likely involve the mining of extensive databases that couple high-throughput analysis of cancer material with clinical response data. The formation of large international consortia, in combination with liberal data sharing by pharmaceutical companies and academia, will be essential to successfully complete these next steps and to improve the outcome of patients with this disease.

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C.J.A.P. has an advisory role for Servier and Nordic Pharma. M.K. has an advisory role for Servier. L.V. declares no competing interests.