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Current and emerging biomarkers in metastatic colorectal cancer

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ABSTRACT

Background The incorporation of novel biomarkers into therapy selection for patients with metastatic colorectal cancer (mCRC) has significantly improved outcomes. Optimal treatment planning now takes into account diverse characteristics of patients and their tumours to create personalized therapeutic plans.

Discussion This review is split into two sections. In the first section, we review the prognostic and predictive significance of expanded *RAS* mutation testing, *BRAF* mutations, *ERBB2* (HER2) amplification, microsatellite instability (MSI) and deficient mismatch repair (dMMR) protein, *NTRK* fusions, *PIK3CA* mutations, and MET amplifications. The therapeutic implication of each of those biomarkers for personalizing therapies for each patient with mCRC is discussed. In the second section, we touch on testing methods and considerations of relevance to clinicians when they interpret companion diagnostics meant to guide therapy selection. The advantages and pitfalls of various methods are evaluated, and we also look at the potential of liquid biopsies and circulating tumour DNA (ctDNA) to change the landscape of therapeutic choice and biologic understanding of the disease.

Summary Routine testing for extended *RAS, BRAF,* dMMR or high MSI, and *NTRK* fusions is necessary to determine the best sequencing of chemotherapy and biologic agents for patients with mCRC. Although next-generation sequencing and ctDNA are increasingly being adopted, other techniques such as immunohistochemistry retain their relevance in detection of HER2 amplification, *NTRK* fusions, and dMMR.

Key Words Biomarkers, colorectal cancer, metastatic, next-generation sequencing, ctDNA, anti-EGFR

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INTRODUCTION

For 20 years, fluoropyrimidine doublet chemotherapy combined with either irinotecan (FOLFIRI or CAPIRI) or oxaliplatin (FOLFOX or CAPOX) has been the cornerstone of treatment for metastatic colorectal cancer (mCRC) in the first- and second-line settings[1,2]. Sequencing of those two regimens does not affect overall survival (OS), and therefore, the choice of first-line therapy often depends on physician and patient preference, coupled with patient comorbidities (for example, pre-existing neuropathy)[3]. Although doublet chemotherapy is preferred because of its association with superior progression-free survival (PFS), sequential single-agent therapies have demonstrated similar OS rates and might be appropriate for some patients who are frail or elderly[4–6].

Since the introduction of targeted therapies, there has been an increased appreciation of the molecular stratification required for personalizing treatment. For example, antibodies against the epithelial growth factor receptor ("anti-EGFR") had limited activity in unselected patients, but were shown to have activity in *KRAS* wild-type cancers[7,8]. In the present review, we discuss clinically important alterations that drive treatment selection, including *KRAS* and *NRAS (RAS)* mutations, *BRAF* mutations, *ERBB2* (HER2) amplifications, deficient mismatch repair (dMMR) or high microsatellite instability (MSI-H), *NTRK* fusions, *PIK3CA* mutations, and MET amplification (Figure 1). In the second section, we review some practical and technical considerations to keep in mind when ordering biomarker tests, and we explore the relevance of next-generation sequencing (NGS) and circulating tumour DNA (ctDNA) or liquid biopsies.

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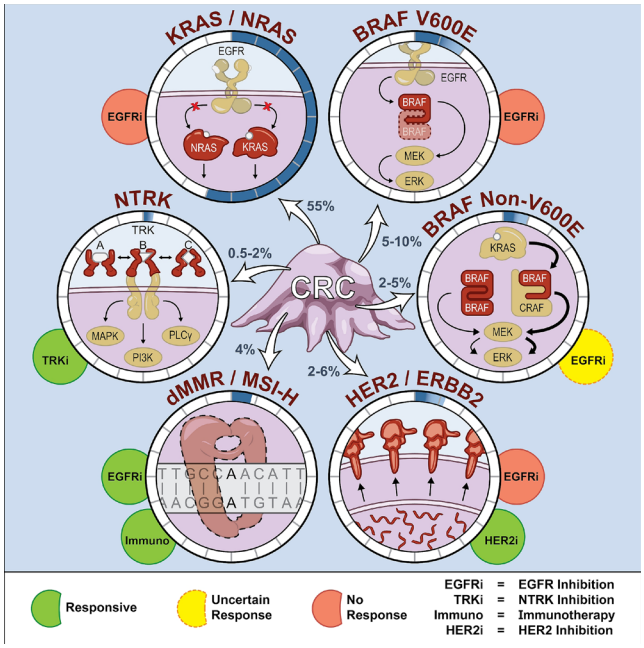

FIGURE 1 Current and emerging biomarkers used in personalizing treatment for patients with metastatic colorectal cancer (CRC). Prevalence of each biomarker in metastatic colorectal cancer is displayed with shading in the circle that surrounds the molecular alteration. Molecular alterations are not mutually exclusive and can co-occur. PLCγ = phospholipase C gamma; dMMR = deficient mismatch repair; MSI-H = high microsatellite instability.

MOLECULAR SUBTYPES IN mCRC

Expanded *RAS* Testing

KRAS codon 12 and 13 mutations were first identified as predictive biomarkers in third-line anti-EGFR trials[7,8]. A subsequent retrospective analysis of the PRIME trial identified expanded mutations in *KRAS* and *NRAS* at codons 12, 13, 59, 61, 117, and 146 as predictive of the ineffectiveness of anti-EGFR therapy[9]. International guidelines now mandate, as the standard of care, expanded *RAS* mutation testing before use of anti-EGFR to identify the 55% of patients with mCRC for whom those agents will be ineffective[9,10]. There is even a suggestion of possible harm with the use of anti-EGFR therapy in patients with *RAS* mutations[11]. Expanded *RAS* mutations are also a negative prognostic marker in the metastatic setting [median OS (mos): 25 months vs. 32.1 months in wild-type disease; hazard ratio (HR): 1.52; 95% CI: 1.26 to 1.84; $p < 0.001$][12]. Compared with *KRAS, NRAS* might be associated with shorter disease-free survival (33 months vs. 47 months; HR: 2.0; 95% CI: 1.3 to 2.8; $p < 0.01$) in early-stage disease and worse OS in mCRC (HR: 1.83; 95% CI: 1.40 to 2.39; $p < 0.001$)[13,14].

Effect of Primary Tumour Location ("Sidedness") on Anti-EGFR Efficacy

Attention to the relevance of primary tumour location increased after a re-analysis of the Cancer and Leukemia Group B (CALGB) 80405 trial showed that, in treatment-naïve patients with mCRC treated with either FOLFOX or FOLIRI (physician's choice) and randomized to the addition of cetuximab or bevacizumab, no difference in mos was evident between the arms overall. However, survival differences were observed between patients with right- and left-sided tumours (mos: 19.4 months vs. 33.3 months; HR: 1.55; 95% CI: 1.32 to 1.82; $p < 0.001$), and the biologic associated with optimal results varied by side. Patients with left-sided tumours experienced improved outcomes with doublet chemotherapy plus cetuximab (mos: 36.0 vs. 31.4 months); those with right-sided tumours appeared to do better with a first-line doublet plus bevacizumab (mos: 24.2 months vs. 16.7 months; HR: 1.27; 95% CI: 0.98 to 1.63; $p = 0.065$)[15]. Those results were subsequently confirmed in numerous other first- and third-line trials that included anti-EGFR therapy. Even when patients with *BRAF* mutations were excluded and adjustments were made in the right-sided tumour group for a higher proportion of female patients and patients with MSI-H disease, primary tumour sidedness remained influential[16–19].

Although those results were retrospectively identified, the reproducibility of the findings across studies has led to sidedness being accepted in many international guidelines as a predictive biomarker[19,20]. Although a doublet plus anti-EGFR appears to be superior to bevacizumab for left-sided tumours, use of that combination in the first-line setting should be considered in balance with the added toxicity, particularly severe rash (~10%) and refractory hypomagnesemia (3%–7%). Those adverse reactions can have a significant effect on quality of life, but are potentially indicators for treatment efficacy[21]. In addition, compared with bevacizumab, anti-EGFR therapy maintenance strategies are less well established[22]. For right-sided tumours, bevacizumab appears superior to anti-EGFR when combined with doublet chemotherapy in the first-line setting[15]. In the third-line setting, patients with right-sided tumours might derive less benefit from anti-EGFR therapy. However, in this patient population, effective treatment options are limited, and therefore, that inferior efficacy has less effect on treatment planning[23].

Rechallenge with Anti-EGFR Therapy

After progression on an anti-EGFR therapy, *KRAS, NRAS, BRAF,* and *EGFR* ectodomain mutations develop to drive signalling through the MAP kinase pathways despite EGFR inhibition. The mutations provide a short-term selective advantage over other subclones, but are selected against with exponential decay after removal of anti-EGFR; their half-life in ctDNA is 4.4 months[24]. Retrospective studies have shown that, after intervening non–anti-EGFR therapies, patients re-challenged with anti-EGFR antibodies experience an overall response rate (ORR) of 23%. In the same cohort, a wait of more than 2 half-lives from prior anti-EGFR therapy was associated with an increase in the ORR to 32%[24]. Similarly, in the prospective CRICKET trial, patients with evidence of persistent *KRAS* mutations experienced an inferior median PFS (mPFS) of 1.9 months compared with 4 months in patients with *RAS* wild-type disease re-challenged with anti-EGFR (HR: 0.44; 95% CI: 0.18 to 0.98; $p = 0.03$)[25]. Based on ctDNA, appropriate patients might be considered for re-challenge with anti-EGFR therapy; however, further randomized prospective studies are needed.

BRAF

BRAF *V600E Mutations*

The serine–threonine BRAF kinase is found downstream from EGFR in the MAPK (mitogen-activated protein kinase) pathway. Hotspot mutations substituting glutamic acid for valine at codon 600 (V600E) result in kinase activity that is increased by a factor of 130 to 700 compared with that in wild-type *BRAF*[26,27]. *BRAF* V600E occurs in 5%–10% of cases of MCRC. It is nearly always mutually exclusive with *RAS* mutations and appears to predict a lack of, or reduced benefit from, anti-EGFR therapy[9,28,29]. *BRAF* V600E mutations are also a strong negative prognostic marker independent of tumour sidedness (mOS range: 10.5–13.5 months mutated vs. 28.3–30.6 months wild-type; HR: 2.01; 95% CI: 1.49 to 2.71; $p < 0.001$)[9,11,12]. Consideration of more aggressive first-line treatment with FOLFOXIRI (fluorouracil–leucovorin–oxaliplatin–irinotecan) and bevacizumab is therefore advocated by some authors, given that FOLFOXIRI has been associated with the greatest mOS for a first-line regimen in patients with *BRAF* V600E mutations in TRIBE and a small Italian phase II trial (mPFS: 7.5 months and 9.2 months respectively; mOS: 19.0 months and 24.1 months respectively)[30,31]. The TRIBE2 study compared upfront FOLFOXIRI plus bevacizumab with a planned sequential switch of FOLFOX plus bevacizumab to FOLFIRI plus bevacizumab, confirming the superiority of a triplet in *BRAF* V600E–mutant MCRC (preliminary mOS: 27.6 months vs. 22.6 months; $p = 0.033$)[32].

In the second-line setting, single-agent *BRAF* inhibitors yielded disappointing results[33]. Combination approaches with irinotecan, cetuximab, and vemurafenib in the SWOG S1406 phase II trial improved the mPFS (2.0 months vs. 4.3 months; HR: 0.48; 95% CI: 0.31 to 0.75; $p = 0.001$), but did not statistically improve OS (5.9 months vs. 9.6 months, $p = 0.19$) in the context of 48% crossover from the control arm[34]. More recently, the BEACON trial showed superior efficacy for the combination of encorafenib, binimetinib, and cetuximab compared with irinotecan or FOLFIRI plus cetuximab (mOS: 9.0 months vs. 5.4 months; HR: 0.52; 95% CI: 0.39 to 0.70; $p < 0.0001$) in the second or later lines of therapy[35]. Interestingly, the triplet did not appear more active than the combination of encorafenib and binimetinib (mOS: 9.0 months vs. 8.4 months; HR: 0.79; 95% CI: 0.59 to 1.06). The magnitude of benefit seen in the the BEACON trial resembled the estimated OS of 9.1 months for dabrafenib–trametinib–panitumumab in another study[36]. Together, those trials support vertical inhibition of the MAPK pathway as a therapeutic option for *BRAF* V600E–mutant MCRC.

Atypical BRAF *Mutations*

More than 200 non-V600E *BRAF* mutations or atypical *BRAF* mutations have been discovered, with a combined incidence ranging from 1.6% to 5.1%[37–39]. *BRAF* V600E mutations have been defined as class I mutations (active monomer); the atypical mutations are split into classes II and III. Class II *BRAF* mutations are constitutively active dimers and have intermediate activity compared with their class I counterparts[40]. Class III *BRAF* mutations result in a protein with a kinase domain having limited signal transduction activity, but it binds to CRAF and activates ERK signalling in a RAS-dependent manner[41,42]. Prognosis appears to be similar for patients with atypical *BRAF* mutations and those with wild-type *BRAF* MCRC[43]. However, the role of BRAF- and EGFR-directed therapy is still being evaluated, although some studies suggest that those mutations might also show a reduced response to anti-EGFR therapy[44–47].

HER2 Amplification

Amplifications of HER2 occur in 2%–6% of MCRC cases, but are enriched in *KRAS*/*NRAS*/*BRAF* wild-type MCRC, with a prevalence of approximately 13% in some series[48–50]. American Society of Clinical Oncology guidelines and the College of American Pathologists define HER2 amplification as a HER2:CEP17 ratio of 2 or greater, or a copy number variant of 4 or greater[51].

Amplifications of HER2 appears to be a negative predictive marker for anti-EGFR therapy, with mPFS being shorter regardless of line of therapy (2.8 months vs. 8.1–9.3 months depending on the study; HR: 7.05 to 10.66; $p < 0.001$)[52–54]. However, HER2 amplification did not appear to impair the response to other non–anti-EGFR therapies[50]. Dual-targeted anti-HER2 therapy in HERACLES (trastuzumab–lapatinib) and MyPathway (pertuzumab–trastuzumab) both showed an ORR of approximately 30%, with the pertuzumab–trastuzumab combination being associated with durable responses of 5.9 months in the second and subsequent lines of therapy when responses are often less than 5%[49,55,56]. Notably, anti-HER2 therapies appear to be ineffective in patients with *RAS* mutations[49]. Those results have led to the prospective phase II SWOG S1613 study comparing pertuzumab–trastuzumab with irinotecan–cetuximab in HER2-amplified, *RAS* wild-type MCRC treated in the second or later line[57]. Additionally, investigation of neratinib–trastuzumab in a similar setting is ongoing (see NCT03457896 at https://ClinicalTrials.gov/).

Microsatellite Status

dMMR and MSI-H

Deficient MMR leads to nucleotide base insertion or deletion in DNA regions with repetitive elements called "microsatellites," resulting in a MSI-H phenotype. The terms dMMR and MSI-H are often used interchangeably, but dMMR refers to the missing proteins (MLH1, MSH2, MSH6, and PMS2) that are usually detected by immunohistochemistry (IHC), and MSI-H refers to the expanded microsatellites detected when 2 or more of 5 microsatellite loci are unstable in a polymerase chain reaction test[58]. Concordance of IHC staining for MMR protein loss with polymerase chain reaction measurement of MSI-H is greater than 90%[59]. High MSI can also arise from somatic hypermethylation of the *MLH1* promotor, typically caused by aberrant methylation associated with the CpG island methylator phenotype, which might be associated with worse prognosis[60]. More recently, NGS panels used for detecting mutations have been modified to detect MSI with high accuracy and represent an option for multiplexing biomarker tests[61].

The prevalence of MSI-H is 15%–20% in all CRCs, but only 4% in MCRCs[62]. In MCRC, MSI-H was previously associated with a worse prognosis, which could be driven partly by the fact that 20%–34.6% of MSI-H CRCs harbour

BRAF V600E mutations[63,64]. Historically, the median OS for MMR-intact mCRC was 17.9 months (95% CI: 16.2 months to 18.8 months) compared with 10.2 months (95% CI: 5.9 months to 19.8 months) for dMMR mCRC[62]. Recently, MSI-H has shown importance in the metastatic setting because of its role as a tissue-agnostic biomarker for immunotherapy, regardless of germline or somatic origin[65]. Studies of monotherapy with checkpoint inhibitors have reported ORRs of 31%–40%[65,66]. With combination nivolumab–ipilimumab, the ORR increased to 55%, with 71% of patients being free from progression at 12 months, with only moderate increases in grade 3/4 toxicities (to 32% from 20%)[64].

Microsatellite Stable mCRC and Immunotherapy

To date, immunotherapy has shown little activity in microsatellite-stable mCRC. Single-agent pembrolizumab and the combinations atezolizumab–cobimetinib (IMblaze 370) and 5-fluorouracil–atezolizumab–bevacizumab (MODUL) have all lacked activity in microsatellite-stable mCRC[65,67,68]. In contrast, the Canadian Cancer Trials Group CO.26 trial showed that combination durvalumab–tremelimumab in microsatellite-stable mCRC was associated with a modest improvement in mOS to 6.6 months from 4.1 months (unadjusted HR: 0.70; 90% CI: 0.53 to 0.92; $p = 0.03$)[69].

In patients with non-hypermutated mCRC, PD-L1 has not been a useful biomarker to date[66,67]. In the Canadian Cancer Trials Group CO.26 trial, retrospective analysis suggested that a high tumour mutational burden (≥28 mutations per megabase) from ctDNA analysis might select patients (21% of the cohort) most likely to benefit from immunotherapy (HR: 0.34; 90% CI: 0.18 to 0.63; $p = 0.004$, with $p = 0.07$ for an interaction test of tumour mutational burden as a predictive biomarker)[70]. Comparatively, in CALGB 80405, a tumour mutational burden of 8 or more mutations per megabase was a positive prognostic marker associated with improved mOS (33.8 months vs. 28.1 months; HR: 0.73; 95% CI: 0.57 to 0.95; $p = 0.020$) in the first-line setting for patients receiving doublet chemotherapy and a biologic[12,71]. In several other studies, higher tumour mutational burden also appears to select patients with MSI-H who are most likely to benefit from immunotherapy. However, the optimal threshold has yet to be defined, even in other cancer types (for example, lung cancer), where immunotherapy is more commonly used[70,72,73].

NTRK Fusion

The *NTRK* genes encode 3 receptors—TrkA, TrkB, TrkC—recently identified as important partners in fusion events observed in multiple cancers that now have effective targeted therapies[74]. The prevalence of *NTRK* fusions is estimated to be 0.5%–2.0% in mCRC, but is enriched to 4% in MSI-H mCRC[22,74–76]. *NTRK1, 2,* and *3* are normally involved in the development and function of the peripheral and central nervous system[77–79]. Targeted DNA and RNA panels, RNA sequencing, florescence *in situ* hybridization (FISH), and IHC are all options for detecting the fusions[80,81]. Larotrectinib and entrectinib are both U.S. Food and Drug Administration–approved Trk inhibitors with ORRs of 75% and 57.4% respectively in more than 10 tumour types. Median duration of response to larotrectinib was not reached after 8.3 months of follow-up, with 55% of patients being free from progression at 1 year, and median duration of response to entrectinib was 10.4 months (95% CI: 7.1 months to not reached)[82,83]. The second-generation *NTRK* inhibitor LOXO-195 yielded an ORR of 34% in 11 tumour types after patients had progressed on first-line Trk inhibitors and could represent a second-line option[84].

PIK3CA Mutations

Activation of the epidermal growth factor receptor leads to signalling through both the MAPK pathway and the PI3K pathway involved in cell survival and proliferation[8]. Intuitively, mutations in *PIK3CA* would result in resistance to anti-EGFR agents, but mutations in exon 9 (68.4% of all *PIK3CA* mutations) have no effect on anti-EGFR efficacy, and mutations in exon 20 (20.4% of *PIK3CA* mutations) have been associated with a lesser response to anti-EGFR therapy only in select studies[85]. Exclusion of patients with *PIK3CA* mutations did not change the response to cetuximab in patients with *KRAS*/*NRAS*/*BRAF* wild-type tumours in CALGB 80405[12]. Meta-analyses support the indeterminacy of an overall effect of *PIK3CA* mutation (regardless of exon), the interpretation of which is confounded by the fact that *PIK3CA* is often a co-mutation with others[86,87]. Currently, there is no clinical implication for *PIK3CA* mutations outside of a research context.

MET Alterations

The MET receptor tyrosine kinase can be overexpressed, mutated, and amplified in mCRC, and its amplification is recognized as a potential mechanism of acquired resistance for mCRC treated with anti-EGFR therapy[88]. However, multiple trials with various forms of MET inhibition have been unsuccessful in mCRC[89]. Therefore, although genomic aberration in *MET* is commonly observed in mCRC, it remains in the research setting and has not been associated with innate resistance to anti-EGFR therapy.

Consensus Molecular Subtyping

As defined by gene expression profiling, mCRC has been divided into 4 distinct consensus molecular subtypes (CMSs). The 4 subtypes are CMS1, with MSI and immune activation (14%); CMS2, with canonical CRC alterations (37%); CMS3, with metabolic dysregulation (13%); and CMS4, with mesenchymal features (23%)[90]. Those subtypes reflect distinct biology and have been shown to be both prognostic for OS and, in the CALGB 80405 trial, predictive for benefit from cetuximab and bevacizumab[91]. Patients classified as CMS1 appear to derive more benefit from bevacizumab, while those classified CMS2 appear to derive more benefit from cetuximab (p for interaction: <0.001). Although many prospective trials using CMS to stratify patients are planned, CMS subtyping is not currently a standard of care and remains in the research environment.

UNDERSTANDING THE TESTS ORDERED

As the number of biomarkers of relevance discovered in mCRC increase, understanding the appropriate methods for identifying abnormalities becomes increasingly important to clinicians. In the second part of this review,

we discuss the relevance of each technique in the era of precision oncology.

Conventional Laboratory Techniques

IHC

Widely available and low in cost, IHC is a method of detecting one or more specific antigens on a tissue by labelling them with an antibody against the whole protein or smaller epitopes. A secondary antibody is applied which is conjugated with either florescent or non-fluorescent markers (such as the brown 3,3′-diaminobenzidine stain commonly seen) that identify the antigen of interest under microscopy[92]. Immunohistochemistry remains an effective method for identifying dMMR and Lynch syndrome[93]. Similarly, the European Society for Medical Oncology recommends a 2-tier *NTRK* fusion detection algorithm with IHC population screening and confirmatory sequencing to reduce cost and handling time[94]. *BRAF* V600E mutation in melanoma can also be determined by IHC with high concordance. However, a high false-positive rate of 39% and a false-negative rate of 11% have been cited in the setting of mCRC[95]. Although other studies suggest better sensitivity and specificity, the conflicting evidence should caution against reliance on IHC alone for the detection of *BRAF* V600E mutations in mCRC[96].

FISH

In the FISH cytogenetic technique, florescent DNA probes are hybridized to a sample, and the relative distribution of the probes determines the cytogenetic defect[97]. Increased numbers of FISH probes can be used as confirmatory testing for HER2 amplification when IHC shows intermediate staining[98]. Detection of fusions by FISH (merging of two probes to give a different colour) is also a possibility, but can be labour-intensive if there are multiple potential fusion partners that are not known. In cases of fusions (such as *NTRK,* with its multiple fusion partners), targeted RNA sequencing is therefore preferred to FISH because of its high sensitivity, high throughput capability, and requirement for no prior knowledge of the fusion partners involved in the translocation[80,81].

Sequencing Techniques

Sanger Sequencing: Sanger sequencing is a direct gene sequencing technique developed in 1977 to which all modern molecular testing assays are benchmarked. However, it has fallen out of favour because of newer technologies that allow for concurrent testing of multiple genes and greater sensitivity for low-allele-frequency mutations[99]. For example, single-gene companion diagnostic kits for *KRAS* use allele-specific polymerase chain reaction to increase assay sensitivity, allowing for detection of allele frequencies as low as 0.1%, compared with the 10% lower limit of detection with Sanger sequencing[100]. The enhanced sensitivity of newer testing assays results in the identification of up to 20% more patients with a *RAS* mutation, which has significant treatment implications[101]. The minimum detectable allele frequency is an important consideration. The CRYSTAL trial showed that *RAS* mutation frequency as low as 0.1% can predict the ineffectiveness of anti-EGFR therapy[102].

NGS: Next-generation sequencing refers to parallel sequencing reactions for multiple genes and multiple samples concurrently. An example of this technology uses fluorescence in added bases to detect the DNA sequence. After DNA purification and fragmentation, single-stranded DNA is attached to the surface of a flow-cell channel. The single strands are amplified repeatedly to create dense clusters of template DNA. Fluorescently labelled reversible terminator nucleotides, which can be detected by a camera, are added to the DNA in subsequent cycles[103]. Other technologies use techniques such as pH changes when a nucleotide is added to provide sequence information.

NGS Sample Selection: When selecting which sample from a patient to use for sequencing, a few important considerations are necessary. In mCRC, high concordance for *RAS* and *BRAF* V600E mutation detection is observed between the primary tumour and metastases at various sites in the body[104–106]. Guidelines therefore suggest that testing a metastatic lesion is ideal; however, archival tissue can be used[10]. Fresh biopsies of recurrent tumours have the advantage of yielding higher-quality nucleic acids, particularly RNA for transcriptomic analysis. Formalin-fixed, paraffin-embedded archival samples more than a few years old can introduce sequencing artefacts or errors[107], but for most non-research biomarkers currently used, such archival samples are acceptable[108].

Sequencing quality also depends on the ratio of tumour to normal tissue in a sample. If a particular nucleotide is sequenced 100 times and only 5% of the sample contains tumour cells, most of the measured DNA will be from normal tissue, making cancer mutations hard to detect. Macrodissection by a pathologist to remove normal tissue under the microscope can help with that challenge. With such dissection, less normal DNA remains to be sequenced, and so more of the sequencing reads are attributed to cancer DNA.

Depth of Coverage: Another important metric in NGS is the concept of depth of coverage. "Depth of coverage" is defined as the number of times a particular nucleotide is sequenced; it is often denoted using a number that represents the average or median of the depth, followed by the letter x (that is, "400x"). With more depth, the variant detected is, importantly, more likely to be real rather than an artefact, and low-allele-frequency mutations are more likely to be detected, although other technical issues can affect sensitivity.

Panel Selection: Selecting the sequencing panel to use can be a challenge for clinicians and patients. At most academic institutions, individual companion diagnostic tests have been replaced by either a "home brew" NGS panel designed to meet local needs or a commercially available NGS panel such as FoundationOne CDx [324 genes (Hoffmann–La Roche, Mississauga, ON)] or Caris Molecular Intelligence [592 genes (Caris Life Sciences, Irving, TX, U.S.A.)]. For routine mCRC management, only a very targeted NGS panel is essential, but to balance the research interests of the institution and the ability to use one panel for multiple types of cancer, larger panels are often performed at a marginal increase in cost (for better economy of scale).

RNA Sequencing: Sequencing of RNA involves extraction of RNA, enrichment for messenger RNA, and subsequent reverse transcription to create complementary DNA that is then sequenced using NGS technologies just as DNA would be. Other technologies such as array-based platforms are available for targeted assessment of gene expression. Apart from its previously mentioned role in fusion detection, RNA sequencing can characterize the differences in gene expression that define the molecular subtypes of mCRC. Several iterations of those molecular subtypes have been presented; the CMS version appears to be of greatest utility, but it remains in the research realm[90]. Sequencing of RNA usually provides information about gene expression, but it can also be used to identify various types of mutations beyond fusions, such as single-nucleotide variants. However, because of sample degradation and artefacts, sequencing of RNA can be more challenging to perform than conventional DNA sequencing in archived formalin-fixed tissues.

Utility of Liquid Biopsy: When discussing liquid biopsies, the term "cell-free DNA" refers to all DNA found in the plasma, of which ctDNA is the subset of tumour origin only. Typically, ctDNA consists of short DNA fragments approximately 166 bp long, with an estimated half-life of 16 minutes to 2.5 hours[109]. In treatment-naïve patients with mCRC, diagnostic molecular profiling of ctDNA appears to have high concordance with tissue-based assays[110]. Although up to 15% of samples can be nondiagnostic because of a lack of detectable ctDNA, the same problem can occur in tissue sequencing[110,111]. Detection of ctDNA has also shown value in the risk-stratification of patients at risk of recurrence. In one study, the HR for stage II patients with detectable ctDNA after resection was 18 (95% CI: 7.9 to 40.0; $p < 0.001$)[112]. Amplifications can also be detected using ctDNA. In the HERACLES trial, ctDNA successfully detected amplifications in 46 of 48 patients with HER2-amplified disease, and detection of higher copy numbers was associated with ORR and PFS[113]. Some ctDNA assays are also validated for detection of MSI[109].

SUMMARY

Increased appreciation of molecular subtypes beyond *KRAS* exon 2–mutant CRC has refined the management of patients with mCRC. With the development of patient-friendly technologies such as ctDNA, which allows for noninvasive molecular assessment, the integration of biomarkers can be expected to become more integral to every decision made in the clinic and will further improve outcomes for patients.

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CONFLICT OF INTEREST DISCLOSURES
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