Chapter

Current Utility and Future Applications of ctDNA in Colorectal Cancer

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Abstract

Circulating tumour DNA (ctDNA) shows promise as a minimally invasive biomarker with a myriad of emerging applications including early detection and diagnosis, monitoring of disease and treatment efficacy, and identification of actionable alterations to guide treatment. The potential utility of ctDNA in colorectal carcinoma (CRC) is of particular interest given the limitations of current radiographic imaging and blood-based tumour markers in detecting disease and evaluating therapeutic benefit. While ctDNA has yet to demonstrate clinical utility in CRC, a growing body of research highlights the potential of these novel biomarkers. This chapter provides an overview of the current evidence for employing ctDNA in CRC as well as previewing the future directions that these exciting technologies may take.

Keywords: colorectal carcinoma/cancer, circulating tumour DNA, biomarker

1. Introduction

Ongoing research in oncology aims to generate patient-directed treatment options, targeting each individual's specific cancer molecular profile with therapies most likely to initiate and maintain an effective anti-tumour response [1]. Currently, molecular profiling in colorectal cancer (CRC) relies on direct biopsy of tumour tissue. However, tissue biopsy presents a number of procedural and biological challenges. Firstly, it is an inherently invasive procedure, making recurrent sampling difficult. Secondly, results may be affected by bias owing to tumoural heterogeneity. Tumours are affected by factors such as genomic instability, the surrounding tissue microenvironment and therapeutic effects [2]. These influences create dynamic molecular selection and evolution, resulting in spacial and temporal heterogeneity, which cannot be represented by a single site tissue biopsy, particularly in the case of metastatic disease [3].

Recognition of these limitations has prompted an interest in non-invasive circulating tumour-specific biomarkers. The concept of 'liquid biopsy' originally described the detection and analysis of circulating tumour cells (CTC) in blood, with reference to tissue biopsy. More recently, it has been broadly adapted to describe any tumour-related constituents circulating in body fluids such as CTC, DNA, RNA and exomes [4]. Compared with tissue biopsies, liquid biopsies may

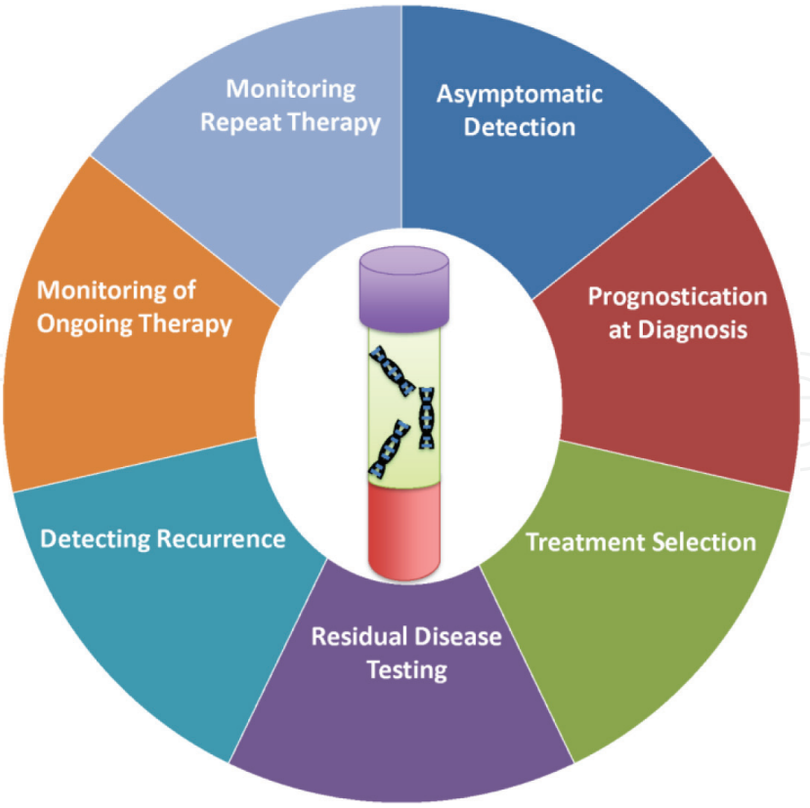

Figure 1.
Emerging ctDNA applications in various CRC management settings.

be better suited for serial surveillance, by reducing procedural time and potential harm. Blood sampling may also provide a more accurate representation of global tumoural heterogeneity, not limited to the site-specific characteristics detected through tissue biopsy [5]. The focus of this review will be directed towards circulating tumour DNA (ctDNA) found in blood samples, which at present, of all the liquid biopsy approaches, has had the greatest clinical impact. CtDNA is thought to be released by tumour cells, containing tumour-specific genetic and epigenetic alterations [6]; and has been found to correlate with tumour stage, burden of disease and response to therapy in CRC [7].

Herein, we provide an overview of ctDNA technologies in use and highlight the emerging clinical applications of ctDNA in various CRC management settings (**Figure 1**). The future directions of this rapidly advancing field will also be explored.

2. ctDNA methodological approaches and technical considerations

Circulating cell-free DNA (cfDNA) was first detected in 1948 by Mendel and Metais in the peripheral blood plasma of healthy and diseased individuals [8]. CfDNA levels can vary between 1 and 10 ng mL^{-1} in plasma and can be affected by physiological conditions such as exercise and acute inflammation [9]. In 1977, Leon et al. found that cfDNA was more elevated in cancer patients compared with healthy subjects, with higher levels correlating with higher burden of disease [10]. In 1989, Stroun et al. discovered that at least part of the plasma DNA in cancer patients originated specifically from cancer cells [11]. In the ensuing decades, knowledge and applications of tumour-derived cfDNA has rapidly evolved due to advances in molecular techniques, and also gave rise to the term, circulating tumour DNA (ctDNA).

A variety of tumour-specific molecular alterations may be identified by ctDNA including mutations, methylation variants, microsatellite alterations, copy number variations and structural changes [12]. Although the exact mechanisms are yet to be elucidated, ctDNA is thought to be released into the blood stream via biological processes such as apoptosis, necrosis, inefficient phagocytosis and active secretion [13, 14]. CtDNA has a short half-life of up to a few hours and accounts for generally only a small fraction of cfDNA, although concentration can vary widely from <0.01 to 90% [12]. The biological and tumoural determinants underlying ctDNA variations both between and within individuals are incompletely understood, but are likely affected by tumour burden, treatment response, circulatory elements, circadian rhythm, cellular turnover and clearance mechanisms [12, 15]. Somatic variants may also be found in healthy individuals, mostly commonly associated with clonal haematopoiesis [5]. Such variability, coupled with the often-low allele frequency of the molecular aberration of interest, demand sensitive and robust detection methods. As we interpret the results of ctDNA studies and consider their clinical relevance, it is prudent to reflect on these biological variables.

2.1 Pre-analytic considerations

Numerous inherent challenges have affected the development of ctDNA pre-analytic and analytic methods. These include variable fragmentation, low abundance in plasma or serum volumes, tumour heterogeneity, and low stability as a result of the aforementioned biological factors [16].

To minimise sample degradation and optimise stability, a number of pre-analytical steps need to be carefully planned. Although there are currently no standardised methodology guidelines on ctDNA collection, storage and processing, the typical workflow is illustrated in **Figure 2**:

2.2 Detection methods

A variety of methods for detecting and characterising ctDNA have been reported. These can be broadly categorised as targeted and non-targeted approaches. Differing performance characteristics, strengths and disadvantages may also facilitate complementary roles of these approaches in molecular analysis. **Table 1** lists examples of described methods. Applying any of these approaches in

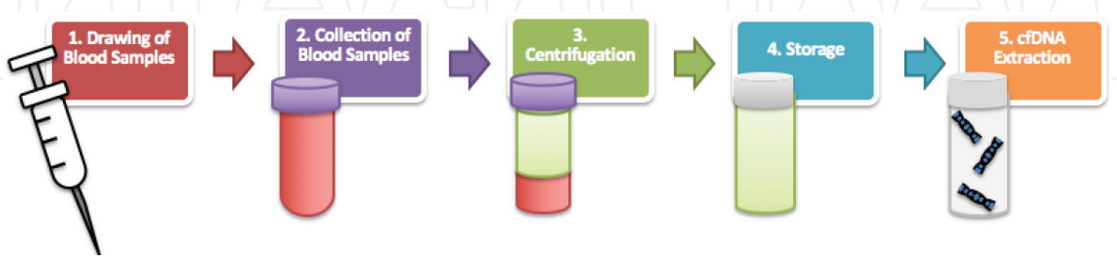

Figure 2.
Pre-analytical components in ctDNA analysis. (1) Collection of blood samples (usually 5–10 mL) via phlebotomy. Currently, there is no guidance on the comparative impact of the source of blood draw (for example, peripheral venepuncture or intravascular ports) on ctDNA analysis [5]; (2) samples should be collected in tubes containing anticoagulants compatible with polymerase chain reaction methods, such as ethylenediaminetetraacetic acid (EDTA) [9]; (3) centrifugation of blood to separate cells should be performed promptly. The exact optimal time to centrifugation is not known and may depend on storage conditions and the presence of stabilising agents [16]. Current evidence suggests that plasma is preferred to serum samples, as in the latter case, cfDNA released during white blood cell lysis may lead to a dilutional effect [9]. (4) Processed plasma is then generally stored frozen, often in aliquots; (5) CfDNA is extracted using commercially available kits. There are multiple DNA purification strategies and modifications, which may variably impact on DNA yield and purity [5].

routine clinical practice in a credentialed laboratory would require considerable scaling up, standardisation, and optimisation of methodological efficiency and accuracy, while minimising cost [5].

Earlier approaches such as Sanger sequencing and conventional polymerase chain reaction (PCR)-based methods have limited sensitivity for ctDNA detection, particularly for rare alterations [6]. A number of digital PCR-based approaches were subsequently developed, capable of improved limits of detection (up to 0.001%), low frequency allele detection and nucleic acid quantitation. Commonly used digital PCR methods for ctDNA detection include BEAMing (beads, emulsion, amplification and magnetics) and droplet digital PCR (ddPCR). BEAMing which combines beads in emulsion and flow cytometry, was first described in 2003 [17] and facilitated serial tracking of mutant allele fractions in patients with CRC [9, 18]. This method and its variations are now widely applied in ctDNA analysis. DdPCR involves the massive partitioning of nucleic acids into thousands of droplets to enable highly sensitive and precise detection and quantitation of small concentrations of DNA [19, 20].

Next generation sequencing (NGS) or massively parallel sequencing are broad terms describing a range of high throughput methods capable of the simultaneous analysis of thousands to millions of DNA molecules, and also encompasses both targeted and non-targeted approaches. Targeted sequencing platforms such as safe sequencing system (Safe-SeqS) [21] and tagged-amplicon deep sequencing (TAm-Seq) [22] have the advantage of improved multiplex capability and evaluating a larger number of loci simultaneously in the genomic areas of interest [15]. However, targeted PCR-based or NGS methodologies mostly rely on prior knowledge of molecular changes and cannot identify variants located in areas that are not analysed.

In contrast, non-targeted genome or exome-wide sequencing allows discovery of *de novo* alterations as well as detection of structural changes including rearrangements, gene fusions and copy number alterations [23]. These may be advantageous in patients who do not have accessible tumour tissue for biopsy. Several techniques have been described and used in the ctDNA setting. The personalised analysis of rearranged ends (PARE) method uses paired-end sequencing, and was utilised in a proof-of-principle study to identify unselected genome-wide chromosomal alterations characteristic of tumour DNA in cfDNA in patients with CRC and

Technique types	Technique	Application
PCR-based [26–28]	ARMS-PCR Mutant allele-specific PCR Bi-PAP	Known point mutations
Digital PCR [18–20, 29]	BEAMing DdPCR	Known point mutations
Targeted sequencing [21, 22, 30]	Safe-SeqS TAm-Seq CAPP-Seq	Point mutation Structural changes in specific gene regions
WGS and WES [24, 31, 32]	PARE Digital karyotyping	Copy number variations Structural rearrangements

PCR, polymerase-chain reaction; ARMS, amplification-refraction mutation system; Bi-PAP, bidirectional pyrophosphorolysis-activated polymerisation; BEAMing, beads, emulsion, amplification and magnetics; ddPCR, droplet digital PCR; Safe-Seq, safe-sequencing system; TAm-Seq, tagged-amplicon deep sequencing; WGS, whole-genome sequencing; WES, whole-exome sequencing; PARE, personalised analysis of rearranged ends.

Table 1.
Methods of ctDNA detection.

breast cancer, including copy number changes and rearrangements [24]. Another group demonstrated the feasibility and utility of exome-wide sequencing in ctDNA to identify mutations associated with acquired therapeutic resistance in a small cohort of patients with advanced cancer [25]. Current limitations of non-targeted approaches include lower sensitivity and relatively prohibitive costs impeding routine clinical implementation [24]. Furthermore, due to the relatively large amount of resulting sequencing data, substantial bioinformatic expertise and filters are required to decipher somatic tumoural alterations from the structural variants commonly seen in germline DNA to avoid false positives [15].

3. CtDNA in screening

Five-year survival for CRC patients is highly dependent on the timing of disease detection and tumour stage. CRC screening can achieve early disease detection and treatment, including that of pre-malignant dysplastic lesions, and has been shown to improve CRC-related mortality. However, 60–70% of patients are diagnosed at mid- to late stage CRC despite recent advances in screening methods [33]. Screening approaches used to test asymptomatic people for a presence of unsuspected disease, which have proven efficacy in CRC include endoscopic visualisation and faecal occult blood tests (FOBT). The former is invasive and expensive with associated morbidity, thus affecting patient compliance and acceptance. FOBT measure the presence of haemoglobin in faeces and can be categorised into guaiac-based (gFOBT) and the newer haemoglobin-antibody-based faecal immunochemical test (FIT). FIT is used more commonly in current practice and has largely superseded gFOBT, due to its superior sensitivity for colorectal bleeding, improved analytical characteristics and it is also less likely to be affected by dietary and medication factors [34–37]. In addition, FIT has better acceptance and participation which improves population participation [38]. In a meta-analysis of 19 studies, FIT was found to have high accuracy and specificity, and moderately high sensitivity, although substantial heterogeneity was noted across studies [39].

This prompted the development of faecal-based tests targeting genetic and epigenetic alterations. Cologuard is the first commercially-approved faecal test which combines several technologies including molecular assays for aberrant *NDRG4* and *BMP3* methylation, *β-actin* (a reference gene for human DNA quantity), and *KRAS* mutations; and a haemoglobin immunoassay [40]. The haemoglobin component of the Cologuard test contributes to 80% of the cancer detection in the algorithm. A large randomised clinical trial comparing Cologuard and FIT screening showed that the sensitivity of Cologuard was superior to that of FIT in the detection of CRC and precancerous lesions [40]. However, the higher cost of Cologuard and its lower specificity compared with FIT has limited its adaptation as a screening tool [33].

CtDNA analysis may offer a more convenient screening approach compared with faecal-based tests. The malignant transformation pathway, from adenoma to carcinoma, is driven by mutations such as *APC*, *KRAS* and *TP53* [41]. However, somatic mutational profiles are highly variable between patients. For example, *KRAS* and *BRAF V600E* are seen in approximately 40 and 7% of patients with CRC respectively [42]. To date, the vast majority of cancer patients evaluated with mutation-based blood plasma assays have advanced-stage disease. A challenge in early stage disease is the often-minute fraction of ctDNA present in the total circulating DNA—may be as low as <0.01%—which may be below the limit of detection assays [18]. A study which enrolled 170 patients with positive FOBT investigated differences in *KRAS* mutation levels in plasma and tissue samples [43]. The rate of *KRAS* mutations in plasma (3%) was found to be lower compared with

that observed in matched adenocarcinoma and high-grade intra-epithelial neoplasia tissues (45%) [43]. Although this is a small study, the results suggest that either this particular assay is not sufficiently sensitive, or that ctDNA was found at low or undetectable levels in the population tested.

The detection of epigenetic alterations characterised by aberrant DNA methylations is an alternative approach to mutational ctDNA analysis. Aberrant DNA methylation leads to transcription silencing of tumour suppressor genes, occurs early in CRC carcinogenesis, and may be more commonly seen and consistent in cancer patients compared with somatic mutations. Indeed, DNA methylation profiles in plasma have been used in biomarker development to identify emergence of early CRC by several groups [44]. One of the methylated promotors that has gained a lot of interest is the *Septin 9* gene promoter. Methylation in the *Septin 9* promoter demonstrated high sensitivity in preclinical studies and a small clinical cohort [45, 46], however a large prospective screening study demonstrated a sensitivity profile of only 48% [47]. In addition, the sensitivity to detect advanced adenomas was low (11%) [47]. Despite this, in 2016, the FDA approved the use of the Epi proColon, a plasma-derived *Septin 9* methylation assay, for the screening of CRC. This decision occurred in the setting of encouraging results from a meta-analysis comparing the pooled sensitivity of methylated *Septin 9* with FOBT as a screening tool [48], and the improved sensitivity and specificity results of a modified version of the Epi proColon assay (2.0 version) [49, 50]. Recently, promising results have been reported in the utilisation of two methylation markers in the screening context—*branched-chain amino acid transaminase 1* (*BCAT1*) and *ikaros family zinc finger protein 1* (*IKZF1*)—where methylation of either gene identified close to 70% of CRC with specificity of 92% [51, 52].

To date, ctDNA is yet to demonstrate clinical utility in the CRC screening setting. Challenges lie in minimising false positive readings, whilst developing a test sensitive enough to detect small amounts of ctDNA. For example, normal physiological ageing is associated with the development of somatic mutations in the absence of malignant disease, and false positive readings may also be seen in patients with chronic inflammatory disease. False positive results can lead to unnecessary follow-up procedures and anxiety. Studies examining a large number of healthy control individuals will be essential to evaluate the specificity of potential screening assays. Yet another challenge with ctDNA-based screening is the identification of the underlying tissue of origin. Because the same gene mutations drive multiple tumour types, ctDNA tests based on genomic analysis alone generally cannot identify the anatomical location of the primary tumour.

4. CtDNA in detection of residual disease in early stage CRC

Although surgical resection can cure a high percentage of patients with CRC, tumour recurrence occurs in approximately 30–50% of all patients who undergo curative resection. The majority of these recurrences take place during the first 2 years after surgery and 90% recur within 5 years [53, 54]. Recommendation for adjuvant cytotoxic therapy is based on clinicopathological risk, although it may not be necessary in many patients and toxicity is substantial. Thus, biomarkers that would aid in identifying patients at high risk of recurrence and who would benefit from adjuvant therapies is of utmost importance. Carcinoembryonic antigen (CEA), a blood protein-based tumour marker, is currently used for monitoring CRC treatment and can also be detected at elevated levels in pancreatic, gastric, lung and breast cancers, as well as a number of benign conditions. Whilst CEA is upregulated in the majority of advanced

CRC, the sensitivity for recurrence detection has been shown to be unacceptably low, approximately 30% [55, 56], supporting the need for alternative markers.

It is well known that in CRC, there is high genomic concordance between the primary tumour and its metastases [57]. Therefore, a promising strategy to detect minimal residual disease or even relapsed disease, could be to use ctDNA to track and quantify key genomic aberrations (*APC*, *KRAS*, *BRAF* and *TP53*), which are recognised as playing a role in early CRC, and may persist in metastatic disease [18, 58]. Several studies have shown that peri-operative ctDNA detection is associated with higher rate of recurrence and in some cases, poorer overall survival; albeit with varying detection methodology, sensitivity and specificity [59–63]. Additionally, the aforementioned methylation markers *BCAT1 and IKZF1* have also shown promise in a study of patients with resected stage I–IV CRC, where post-operative positivity for *BCAT1/IKZF1* methylation was more sensitive (68%) for recurrence detection than CEA (32%, $p < 0.05$) and its odds of recurrence given a positive test (14.4, 95% CI: 5–39) was twice that of CEA (6.9, 95% CI: 2–22) [64]. However, they fail to detect advanced adenomas despite their frequent presence in cancer and adenoma tissue [65]. It would be reasonable to speculate that the release of any DNA from neoplasia seems to be a function of progression along the oncogenesis pathway and it is not a simple reflection of whether or not the change is present in tissue.

These preliminary studies support the need for large prospective trials evaluating pre- and post-operative ctDNA-based biomarkers to help predict recurrence and evaluate prognosis. However, it is not yet known whether ctDNA represents the molecular diversity of disease or whether only selective clones (for example, highly apoptotic clones) are secreted into the bloodstream. Furthermore, it is yet to be established whether early detection of recurrence can indeed improve survival outcomes, if treatment is followed soon after.

5. CtDNA in metastatic CRC (mCRC)

For the majority of patients with mCRC, the mainstay of treatment consists of palliative-intent systemic therapy with median overall survival (OS) approaching 24 months. Three classes of cytotoxic agents (fluoropyrimidine, irinotecan and oxaliplatin) and two classes of molecularly-targeted agents (monoclonal antibodies targeting vascular endothelial growth factor [VEGF], and the epidermal growth factor receptor [EGFR]) are currently approved for use in mCRC, although the optimal sequencing and scheduling of these treatments are debated. To optimise their therapeutic ratio and minimise toxicity, effective and accurate means of assessing treatment response are needed. In the following section, we summarise the evidence on the use of ctDNA in mCRC management. These include prognostication, monitoring tumour burden and predicting treatment efficacy, guiding targeted treatment selection, and detecting anti-EGFR therapy resistance.

5.1 CtDNA in mCRC: prognostic value and monitoring tumour burden

The association between the presence or high levels of ctDNA and adverse survival outcomes in mCRC has been demonstrated in several studies [66–69]. For example, in a landmark study, Bettegowda et al. observed a steady decrease in 2-year survival rate as ctDNA concentration increased [69]. Moreover, a systematic review exploring the prognostic role of ctDNA in CRC (mostly mCRC) found that most studies, although not all, demonstrated a negative correlation between ctDNA and disease-free survival and OS [70].

Another application for ctDNA that has been explored in mCRC is correlating longitudinal dynamics during systemic therapy with prediction of treatment response and tumour burden [71]. Currently, anatomical radiographic imaging—particularly computed tomography (CT)—is the chief modality to evaluate therapeutic benefit in mCRC. However, limitations include cost, operator- and reader-dependence, challenges in standardisation and radiographic lag behind clinical changes. Furthermore, changes in tumour size which form the basis of response measurement on CT does not account for changes in tumour density or morphology that may result from response to molecularly targeted agents commonly used in mCRC. CEA is also used in mCRC disease monitoring, usually in-between or in addition to radiology assessments. However, CEA is elevated in only approximately 70–80% of patients with mCRC and has limited sensitivity and specificity in detecting disease progression or treatment response [72].

A study of 53 mCRC patients undergoing standard first-line chemotherapy, found that significant decline in ctDNA levels using Safe-SeqS prior to cycle two chemotherapy was associated with objective radiological response at 8–10 weeks ($p = 0.016$) [73]. This study also found a trend between ctDNA reduction and improved progression-free survival. The more recent PLACOL study in 82 patients receiving chemotherapy for mCRC echoed these findings [7]. PLACOL utilised picodroplet-digital PCR assays based on either genomic or hypermethylation alterations. The investigators found that the baseline ctDNA concentration was prognostic for OS, and that early and deep ctDNA reductions were associated with improved objective response rate and longer survival ($p < 0.001$) [7]. Another recent study using digital PCR found methylation changes over time correlated with tumoural response in patients with mCRC [74].

These studies suggest that early changes in ctDNA during systemic therapy may be predictive for treatment efficacy and prognostic for survival outcomes, thus suggesting a role for serial ctDNA monitoring during palliative treatment with systemic therapy. Indeed, with the advantages of a short half-life reflecting immediate-term changes [18] and high tumour-related specificity, ctDNA monitoring may be complementary to radiological assessments and blood biomarkers currently in use. In clinical circumstances where radiological assessments are indeterminate or ambiguous, such as the lack of measurable disease by imaging criteria or the presence of mixed response, ctDNA dynamics may be of particular value; although ctDNA may not always correspond to imaging findings [75]. Equally, it is also prudent to acknowledge that no current evidence supports the strategy of biomarker-monitoring of palliative therapy and that earlier adaptive treatments will augment survival or quality of life.

5.2 CtDNA in mCRC: genotyping to guide targeted treatment selection

It is now standard of care for patients with mCRC to undergo molecular profiling on their tumour tissue in order to determine *BRAF V600E* and *Rat sarcoma* (*RAS*, particularly exon 2–4 *KRAS*) gene mutational status. This informs clinical decision-making regarding benefit from anti-EGFR therapy. The advantageous role of genotyping with ctDNA has already been established in the field of advanced non-small cell lung cancer. Circulating genetic aberrations of EGFR (for example, exon 19 deletions or exon 21 (L858R) substitution mutations) are now being used in standard practice, as a companion tool, to identify eligible patients for treatment with erlotinib. This technology was approved by the US Food and Drug Administration in 2016 for this indication [76].

In mCRC, a meta-analysis of 21 studies on the diagnostic performance of ctDNA-based *KRAS* gene detection found overall high pooled specificity (96%)

and moderate sensitivity (67%) [77]. Not surprisingly, heterogeneity was noted to be high probably owing to varying molecular techniques, tumour stage and study designs [77]. Although a number of *KRAS* ctDNA assays have demonstrated high agreement (91–93%) with tumour tissue *KRAS* testing and are available for commercial use [78, 79]; at present, tumour tissue testing remains the gold standard to establish *KRAS* mutational status. Given the appreciable discordance rate with tumour tissue genotyping, it is recommended that a negative ctDNA result should trigger tumour tissue variant analysis [5]. As will be discussed under Section 6, ctDNA assays in mCRC may also been utilised to select predictive immune-related biomarkers for immunotherapy selection.

5.3 CtDNA in mCRC: genotyping to monitor for targeted treatment response and resistance

The role of genomic alterations and their evolution in both the development and progression of CRC have culminated in the realisation that serial genotyping of the primary tumour, and its secondaries, is ideally required if we want to succeed in personalising patient care with precision [80]. Unfortunately, patients with mCRC who do not harbour a somatic *RAS* mutation pre-treatment, will typically develop acquired resistance to anti-EGFR therapy in a matter of months after initially showing response. There is a battery of pre-clinical and clinical evidence which points to the acquisition of molecular mechanisms of resistance associated with aberrations in the *RAS-MEK-mitogen-activated protein kinases* (*MAPK*), pathway [2, 42, 81, 82].

Longitudinal ctDNA analysis can be used in this setting with high diagnostic precision to detect both primary resistance and early molecular changes that may confer acquired resistance. Several translational studies have successfully employed ctDNA to illustrate and verify the emergence of *RAS* mutations as a mechanism of acquired resistance to anti-EGFR therapy.

In a phase II trial of anti-EGFR antibody, panitumumab in mCRC, serial prospective plasma analysis detected more emergent *RAS* mutations than serial tissue biopsies, suggesting that the former may be more comprehensive in evaluating global tumoural heterogeneity [83]. In a small retrospective study of 10 mCRC patients who developed resistance to anti-EGFR therapy (cetuximab or panitumumab) in combination with chemotherapy, Misale et al. demonstrated that the onset of the emerging *KRAS* mutations was detected in serum ctDNA analysis as early as 10 months prior to radiological reporting of disease progression [2]. In this study, ctDNA analysis was also explored in a separate cohort of patients who were receiving chemotherapy alone (control group). No acquired *KRAS* mutations were identifiable at disease progression [2]. In the same year, Diaz et al. also demonstrated the feasibility of using serum ctDNA to identify emerging resistance to panitumumab in a prospective cohort of 28 patients [81]. Thirty-eight percent of patients whose tumours were initially *KRAS* wild type developed detectable *KRAS* mutations in their sera, three of whom developed multiple different *KRAS* mutations. These were detected approximately 5 months before radiological progression [81]. Another study subsequently made the intriguing observation that *KRAS* clones can fluctuate under the selective pressure of anti-EGFR therapy implying that there may be a role for 'pulsing' or re-challenging with anti-EGFR therapy [84].

Furthermore, in a prospective study of 108 patients, treated in the third line setting with cetuximab and irinotecan, Spindler et al. investigated the quantitative correlation between plasma cfDNA with tumour-specific plasma mutant *KRAS* levels [85]. This study revealed that (i) the majority of *KRAS* mutations that were detected in tumours were also found in the plasma, (ii) there was a strong correlation between cfDNA and plasma mutant *KRAS* levels, and (iii) high levels of plasma

mutant *KRAS* were associated with 0% disease control rate [85]. More recently, a large retrospective exploratory analysis used BEAMing technology to identify *KRAS*, *PIK3CA* and *BRAF* mutations in the plasma ctDNA of 503 patients who enrolled in the CORRECT trial of regorafenib, a multi-kinase inhibitor in refractory mCRC [66]. Tumour-associated *KRAS* mutations were readily detected with BEAMing of plasma DNA and were identified in 48% of patients who had previously received anti-EGFR therapy and whose archival tumour tissues were KRAS wild type [66].

Beyond *KRAS* mutations and amplifications, acquired genetic aberrations in other genes have been found to potentially lead to anti-EGFR therapy resistance, albeit in smaller subsets of patients. For example, emerging *EGFR* extracellular domain (ECD) mutations which lead to impaired antibody binding were found to be a resistance mechanism to anti-EGFR therapy in approximately 20% of patients. Interestingly, these mutations tend to arise later than *RAS* mutations during therapy, and patients with *EGFR* ECD mutations had greater and more durable response to anti-EGFR therapy than patients with *RAS* mutations [86]. Interestingly, a phase I trial of a third generation EGFR-targeting agent that binds multiple regions of the EGFR ECD demonstrated efficacy in patients with *EGFR* ECD mutations and acquired resistance to prior EGFR blockade [87]. Other genomic alterations linked to acquired resistance to EGFR blockade include *MET and ERBB2* amplifications [88, 89] and mutations in *NRAS*, *BRAF* and *PIK3CA* [6]. *ERBB2* amplification was found in the plasma in four out of eight *RAS* wild type patients who derived no clinical benefit from anti-EGFR treatment, suggesting that it may also be a source of primary resistance [84].

Another innovative study provided proof-of-principle that parallel analysis of patient-derived xenografts and ctDNA allowed the identification of resistance mechanisms to a pan-tropomyosin-related kinase (TRK) inhibitor in mCRC, with validation in preclinical models [90]. In interpreting these translational findings, it is important to note that typically, multiple complex molecular abnormalities emerge rather than a singular clone and an overlap exists between abnormalities associated with primary and secondary resistance [6].

CtDNA genotyping has now paved the way for prospective clinical trials which aim to evaluate a range of targeted agents in mCRC and their resistance mechanisms. However, significant knowledge gaps exist in the field, including lack of standardisation of ctDNA techniques, clinical relevance of minority clones detected (for example, no threshold for *KRAS* allele frequency has been established to predict anti-EGFR therapy resistance) and it remains to be proven that changing treatment strategy according to ctDNA findings improves patient outcomes [6]. Challenges notwithstanding, it is foreseeable that in the near future, ctDNA genotyping may be used longitudinally to (i) identify *RAS* wild type patients with mCRC who may be suitable for anti-EGFR antibodies, (ii) dynamically assess treatment response, (iii) identify patients who are developing acquired resistance, (iv) delineate resistance mechanisms to therapy, and (v) discover new druggable targets.

6. Future directions

Despite growing enthusiasm, ctDNA in CRC remains largely unavailable for clinical application outside of the trial setting. Recently, there has been a surge of research to further investigate the utility of more sensitive and accurate technologies for ctDNA detection and analysis, and to further elucidate its clinical implementation and significance in the various settings of CRC management.

6.1 Advancing ctDNA detection accuracy

Improved sensitivity techniques with the use of targeted-sequencing methods have been developed by several groups [9, 91]. For example, Lanman et al. validated the analytical and clinical use of a novel, ultra-high specific, digital sequencing technique (Guardant360) consisting of 54 clinically actionable cancer genes [91]. In 165 consecutively matched plasma and tumour tissue samples from patients with advanced cancer, this study demonstrated significantly improved sensitivity for Guardant360 in the plasma-derived cfDNA compared to that of tumour tissue. It also demonstrated the clinical success rate of the assay in 1000 consecutive plasma samples in the clinic (assay failure rate of 0.02%) due to its ability to eliminate false positives [91].

Other investigators have combined the use of DNA fragment sequencing by using molecular barcodes with relevant bioinformatics filtering steps to enhance sensitivity and specificity [30, 69, 92, 93]. In a study using cfDNA from mCRC patients, Mansukhani et al. showed that false positive mutation calls could be reduced by 98.6% when incorporating novel molecular barcodes for error correction and by applying custom solution hybrid capture enrichment [93].

6.2 Detecting aberrant DNA methylation

Several studies have explored the use of DNA methylation markers that may have a role in CRC screening and diagnosis, and which in some cases may have similar sensitivity and specificity to the aforementioned *Septin 9* methylation assay (for example, *APC*, *MGMT*, *RASSF2A*, *Wif-1*, *ALX4*, *NEUROG1*) [94–99]. More compelling is the evidence suggesting that the use of a combination of DNA methylation markers—a multigene methylation signature—may enhance sensitivity and specificity compared with single biomarker detection [94, 96]. Such an assay, utilising the methylation of both *BCAT1* and *IKZF1*, has shown promising results in this setting as previously discussed [51, 52, 64, 100].

6.3 Using CTC, extracellular vesicles, and microRNA as adjuncts biomarkers

This chapter has highlighted the recognisable potential for a paradigm shift with the use of ctDNA for the molecular diagnosis and monitoring of CRC, as well as its multiple drawbacks when used in isolation. Notably, ctDNA is largely unable to evaluate biomarkers other than genomic aberrations. An alternative approach is the use of tools such as CTC, extracellular vesicles, and circulating microRNAs (miRNA), in conjunction to ctDNA, to overcome these limitations.

6.3.1 CTC

A significant limitation of utilising CTC as a biomarker in CRC, particularly in early disease, is that they are difficult to detect in the blood due to a large proportion being captured in the liver prior to entering the general systemic circulation [101–103]. Furthermore, there have been a number of heterogenous studies, systematic reviews and meta-analyses which demonstrate conflicting results for the role of CTC as a prognostic biomarker [104–106]. This makes interpretation very challenging. Nonetheless, it is worth noting that detectable ctDNA and CTC as biomarkers are distinct entities and, in isolation, neither can be regarded as optimal surrogates of the multiclonal malignant state in an individual CRC patient. As discussed earlier, ctDNA is likely to be released by apoptotic or necrotic tumour cells, rather than highly proliferative cells, within a multiclonal tumour [103]. However, we do not know whether all clones have the same apoptotic potential, and therefore

detectable ctDNA levels in CRC patients do not always correspond to the ability to detect CTC [102, 107]. As such, it would be worth exploring the concomitant use of both of these biomarkers in a liquid biopsy.

6.3.2 Extracellular vesicles

The clinical utility for these small, membrane-bound cell fragments, which are also thought to originate from apoptotic, necrotic or proliferating tumour cells, has also recently been considered [108]. Depending on their size and content, they fall under the categories of exosomes, microvesicles, and apoptotic bodies. In particular, tumour-derived exosomes are constitutively formed and released from tumour cells and can be found in the peripheral circulation, other body fluids and interstitial spaces. They can contain concentrated forms of RNA, miRNA, long non-coding RNA, nucleic acids, protein and lipids, but only very small amounts of double-stranded DNA [109]. As such, exosome-derived nucleic acids from the serum of CRC patients may be used to identify genetic aberrations from the tumour that are not detectable by ctDNA, and therefore can be used in a complementary fashion with other biomarkers. To date, there is no published data that has alluded to their clinical application in CRC. Intriguingly, exosomes have recently been shown to have roles in cell-cell signalling which may affect tumour growth and development [110].

6.3.3 miRNA

The role of circulating, exosome-free miRNAs as potential diagnostic and prognostic biomarkers in CRC has been extensively investigated over the past 5 years [111–116]. However, owing to extensive heterogeneity between several studies, it has been difficult to gauge their clinical worth in terms of sensitivity and specificity, which has often been described as 'modest'. Encouragingly, recent studies have suggested that by using miRNA panels or signatures, the predictive accuracy of these assays can be significantly enhanced [117, 118]. In mCRC, only a few studies have addressed the role of circulating miRNAs as predictive biomarkers to systemic therapy [119]. Conceptually, miRNA assays could be used in conjunction with ctDNA, or with the aforementioned biomarkers, to facilitate accurate read-outs for improved sensitivity and specificity.

6.4 Detecting microsatellite phenotype

The use of immune-checkpoint inhibitors has drastically changed the therapeutic landscape for several solid tumours, including a mCRC subset that harbours mutations in DNA mismatch repair (MMR) genes (for example, mutations in *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *MLH1* promoter hypermethylation) [120]. On a molecular level, impaired DNA MMR can lead to genomic hypermutability, including uncontrolled expansion or contraction in DNA microsatellite repeats, termed microsatellite instability (MSI); and the consequent development of malignant neoplasms which have an MSI-high (MSI-H) phenotype. The MSI-H phenotype is present in approximately 15% of all primary CRC and may occur as a result of either inherited (hereditary non-polyposis colon cancer or lynch syndrome) or sporadic abnormalities. It is now common for institutions to screen for this in tumour tissue, either by immunohistochemistry for deficient MMR (dMMR) or PCR for MSI [121]. The identification of the MSI-H phenotype in CRC patients has important prognostic and therapeutic implications, both in the adjuvant and advanced settings when considering conventional chemotherapeutic and targeted agents.

More recently, a small phase II clinical trial using pembrolizumab, an anti-programmed cell death protein 1 (PD1) monoclonal antibody in dMMR mCRC

patients, demonstrated high rates of objective response (40%) and progression-free survival, while no responses were seen in proficient MMR patients [122]. Similarly, a phase II study of anti-PD1 antibody, nivolumab in dMMR/MSI-H mCRC showed 31% objective response rate and 69% disease control rate (12 weeks or longer) [123]. In addition to these encouraging results, multiple trials using anti-PD1 agents, with or without other targeted therapies are ongoing (for example, NCT02460198, NCT02563002, NCT02060188), and it is expected that immunotherapy will rapidly become standard of care in dMMR/MSI-H mCRC.

In this setting, liquid biopsy might be useful in providing a potentially faster, cost-efficient, and safer approach compared to tumour biopsy sampling in patients with suspected MSI-H tumours. Therefore, such assays need to be optimised for routine use in the future. The novel ctDNA techniques described earlier in this chapter could be adapted to identify dMMR CRC in several ways, such as change in microsatellite length, loss of heterozygosity, mutations, or hypermethylation of MMR-related genes [124]. However, similar drawbacks regarding their sensitivity and specificity apply in this setting [125]. To overcome these limitations, several groups have developed enrichment techniques which are able to enhance the presence of altered microsatellites with enrichment probes and detect alterations at very low allele frequencies [126].

Moreover, ctDNA in the setting of immunotherapy can also be used (i) as a predictive marker to identify tumour mutational burden or specific response mutations (for example, PTEN loss or activating beta-catenin mutations), (ii) to monitor treatment response or resistance in conjunction with radiological imaging, and (iii) to identify neoepitopes and epigenetic or transcriptomic markers [124]; although the data for such techniques are preliminary at this stage.

6.5 Detecting ctDNA in other body fluids

This chapter has predominantly focused on the utility of ctDNA in the peripheral blood. Multiple studies have also demonstrated the presence of tumour-derived nucleic acids in other body compartments, such as the urine, stool, saliva, cerebrospinal fluid, pleural fluid, and bronchial washings [40, 127–129]. Of course, topography of the primary tumour, and of any disseminated lesions, will have a significant effect on the concentration of ctDNA in different body fluids.

In a small study, Fujii et al. demonstrated the utility of detecting *KRAS* mutations in the urine of mCRC patients who were undergoing systemic treatment. Both NGS and enrichment PCR were used to detect KRAS in the urine, plasma and archival tumour tissue [128]. The results not only suggested good concordance between ctDNA in the urine and mutant *KRAS* in the tumour, but also demonstrated that ctDNA trends in the urine reflected the tumour dynamics in the plasma. As such, this may also represent an alternative approach to monitoring for therapeutic response or resistance.

7. Conclusion

The data generated from basic research, retrospective clinical studies, and limited prospective studies all support the potential role of ctDNA as a biomarker for early disease, minimal residual disease, recurrence, response to therapy, and emerging drug resistance mechanisms in the management of CRC. Nevertheless, multiple challenges need to be overcome before this promising technology can be adopted into routine clinical practice.

Firstly, a crucial question is whether the genomic aberrations detected in ctDNA actually drive tumour progression. It is also still unknown whether ctDNA will ever be able to mirror the heterogeneity or molecular subclones of CRC in a given

Clinical trial identifier	Study title
Curatively treated CRC (recurrence surveillance and prognostication)	
NCT02842203	Use of ctDNA for Monitoring of Stage III Colorectal Cancer
NCT02842203	Circulating Tumour DNA Analysis to Optimise Treatment for Patients With Colorectal Cancer (IMPROVE)
NCT03416478	The Implication of ctDNA in the Recurrence Surveillance of Stage II and III Colorectal Cancer
NCT03312374	ctDNA as a Prognostic Marker for Postoperative Relapse in Early and Intermediate Stage Colorectal Cancer
NCT02997241	Colon Cancer Treatment Decisions and Recurrence Predicting (CCTDRP)
NCT03189576	Measuring Molecular Residual Disease in Colorectal Cancer After Primary Surgery and Resection of Metastases
NCT03038217	Investigation of the Value of ctDNA in Diagnosis, Treatment, and Surveillance of Surgically Resectable Colorectal Cancer
NCT03615170	Application of Circulating Tumour DNA Test in the Diagnosis and Treatment of Patients With Advanced Rectal Cancer
mCRC—monitoring during chemotherapy	
NCT02872779	Correlation Between Circulating Tumour Markers Early Variations and Clinical Response in First Line Treatment of Metastatic Colorectal Cancer (COCA-MACS)
NCT02948985	Evaluation of CTCs Combined With Tumour Marker Detection of Efficacy of Chemotherapy in mCRC
mCRC—RAS testing	
NCT02502656	RAS Mutation Testing in the Circulating Blood of Patients With Metastatic Colorectal Cancer (RASANC)
NCT03227926	Rechallenge With Panitumumab Driven by RAS Dynamic of Resistance (CHRONOS)
NCT03259009	RAS Mutations in ctDNA and Anti-EGFR reINTROduction in mCRC (RASINTRO) (RASINTRO)
mCRC—MSI testing	
NCT03561350	Detect Microsatellite Instability Status in Blood Sample of Advanced Colorectal Cancer Patients by Next-Generation Sequencing
NCT03594448	Detection of MSI in Circulating Tumour DNA of Colorectal Carcinoma Patients
Large multi-disease observational studies	
NCT03517332	Circulating Tumour DNA Exposure in Peripheral Blood
NCT02838836	Tumour Cell and DNA Detection in the Blood, Urine and Bone Marrow of Patients With Solid Cancers
NCT03027401	Clinical Sequencing of Cancer and Tissue Repository: OncoGenomics
Other	
NCT03546569	Tumour Cells, Tumour DNA and Immunological Response in Colonic Stent Placement (CISMO)
NCT03284684	Kinetics of Perioperative Circulating DNA in Cancer Surgery (Periop ctDNA)
NCT02579278	Circulating Tumour DNA (ctDNA) Rectal Cancer and the Relationship to Extramural Venous Invasion (ctDNA Trial)

Table 2.
Currently recruiting and upcoming clinical trials assessing ctDNA in CRC (http://clinicaltrials.gov).

patient. Further clarity is also needed regarding intra-patient variability in ctDNA levels, the dynamics of ctDNA release and ctDNA clearance. Such knowledge will inform the design of future studies, particularly regarding the optimal timing of ctDNA assessment relevant to the appropriate therapeutic intervention.

Secondly, to determine the true value of ctDNA analysis in guiding decision-making, carefully designed and well-controlled prospective trials are needed to address clinically relevant questions for various settings. An important question, for example, is how to utilise ctDNA detection as a biomarker of minimal residual disease after resection of a stage I–III CRC. Can we use this biomarker to make decisions about the necessity, type and duration of adjuvant therapy and guide follow-up or surveillance scheduling? Another question is how to use ctDNA to monitor for the emergence of molecular resistance and can we use this approach to inform us about timely adaptation of further treatment lines? **Table 2** lists selected currently recruiting and upcoming clinical trials assessing the utility of ctDNA in various settings in CRC.

Importantly, pre-analytical considerations, ctDNA detection techniques, and interpretation of results need to be standardised. On review of the current literature, it will be obvious to the reader that there is a high level of heterogeneity amongst various techniques. Consequently, results that are obtained from one study cannot be interpreted in the same way and applied to other techniques. Standardisation will ensure that there is consensus regarding the sensitivity and specificity of utilised techniques and that there are established cut-off levels, for each clinical setting. Finally, it is important to acknowledge that the use of promising novel technologies will have cost implications which may hinder their rapid entry into routine clinical practice.

Glossary

Allele frequency	The relative frequency of a gene variant in a specimen, expressed as a percentage or fraction
CfDNA	Cell-free DNA. DNA fragments found circulating in body fluids, including plasma or serum. CfDNA may come from a variety of sources including tumour cells
CtDNA	Circulating-tumour DNA. A proportion of cfDNA that is tumour-derived
Liquid biopsy	Sampling and analysis of tumour-based material (e.g. CTC, ctDNA, RNA, exosomes) from body fluids such as blood, urine and pleural fluid
NGS	Next generation sequencing (NGS) or massively parallel sequencing are broad terms describing a range of high throughput methods capable of the simultaneous analysis of thousands to millions of DNA molecules
PCR	Polymerase chain reaction. A laboratory technique used to make many copies (amplification) of a specific DNA sequence of interest
Pre-analytical	The pre-analysis phase in the laboratory testing process and may include sample collection, handling, processing, transport and storage. These factors can affect the subsequent analysis outcomes
Clinical utility	The ability of an intervention or test to demonstrate benefit in patient care compared to not using the intervention or test

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