Chapter

Current Utility and Future Applications of ctDNA in Colorectal Cancer

Daphne Day, Sophia Frentzas, Cameron A. Naidu, Eva Segelov and Maja Green

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 spatial 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
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

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**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].

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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           | Known point mutations           |
|                          | Mutant allele-specific PCR |                                  |
|                          | Bi-PAP             |                                  |
| Digital PCR [18–20, 29]  | BEAMing            | Known point mutations           |
|                          | DdPCR              |                                  |
| Targeted sequencing      | Safe-SeqS          | Point mutation                   |
| [21, 22, 30]            | TAm-Seq            | Structural changes in specific gene regions |
|                          | CAPP-Seq           |                                  |
| WGS and WES [24, 31, 32]| PARE               | Copy number variations           |
|                          | Digital karyotyping| 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.
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 methylation 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 pro-Colon, 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 Ras 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 be 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 relINTROduction 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

| Term  | Definition |
|-------|------------|
| 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 |
Author details

Daphne Day¹², Sophia Frentzas¹², Cameron A. Naidu²³, Eva Segelov¹² and Maja Green²*

1 Department of Medical Oncology, Monash Health, Clayton, Victoria, Australia
2 Department of Oncology, Monash University, Clayton, Victoria, Australia
3 Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton, Victoria, Australia

*Address all correspondence to: maja.green@monash.edu
References

[1] Li ZZ et al. Mutation profiling in Chinese patients with metastatic colorectal cancer and its correlation with clinicopathological features and anti-EGFR treatment response. Oncotarget. 2016;7(19):28356-28368

[2] Misale S et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486(7404):532-536

[3] Gerlinger M et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. The New England Journal of Medicine. 2012;366(10):883-892

[4] Alix-Panabieres C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discovery. 2016;6(5):479-491

[5] Merker JD et al. Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. Journal of Clinical Oncology. 2018;36(16):1631-1641

[6] Nadal C et al. Future perspectives of circulating tumor DNA in colorectal cancer. Tumour Biology. 2017;39(5):1010428317705749

[7] Garlan F et al. Early evaluation of circulating tumor DNA as marker of therapeutic efficacy in metastatic colorectal cancer patients (PLACOL study). Clinical Cancer Research. 2017;23(18):5416-5425

[8] Mendel P, Metais P. Les acides nucléiques du plasma sanguin chez l’homme. Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales. 1948;142:241-243

[9] Wan JCM et al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nature Reviews. Cancer. 2017;17(4):223-238

[10] Leon SA et al. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Research. 1977;37(3):646-650

[11] Stroun M et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology. 1989;46(5):318-322

[12] Diaz LAJ, Bardelli A. Liquid biopsies: Genotyping circulating tumor DNA. Journal of Clinical Oncology. 2014;32(6):579-586

[13] Jahr S et al. DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Research. 2001;61(4):1659-1665

[14] Khakoo S et al. Circulating tumour DNA, a promising biomarker for the management of colorectal cancer. Critical Reviews in Oncology/Hematology. 2018;122:72-82

[15] Haber DA, Velculescu VE. Blood-based analyses of cancer: Circulating tumor cells and circulating tumor DNA. Cancer Discovery. 2014;4(6):650-661

[16] Gorgannezhad L et al. Circulating tumor DNA and liquid biopsy: Opportunities, challenges, and recent advances in detection technologies. Lab on a Chip. 2018;18(8):1174-1196

[17] Dressman D et al. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(15):8817-8822
[18] Diehl F et al. Circulating mutant DNA to assess tumor dynamics. Nature Medicine. 2008;14(9):985-990

[19] Hindson BJ et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical Chemistry. 2011;83(22):8604-8610

[20] Sanmamed MF et al. Quantitative cell-free circulating BRAFV600E mutation analysis by use of droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors. Clinical Chemistry. 2015;61(1):297-304

[21] Kinde I et al. Detection and quantification of rare mutations with massively parallel sequencing. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(23):9530-9535

[22] Forshew T et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Science Translational Medicine. 2012;4(136):136ra68

[23] Siravegna G et al. Integrating liquid biopsies into the management of cancer. Nature Reviews. Clinical Oncology. 2017;14(9):531-548

[24] Leary RJ et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Science Translational Medicine. 2012;4(162):162ra154

[25] Murtaza M et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013;497(7447):108-112

[26] Board RE et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Research and Treatment. 2010;120(2):461-467

[27] Yamada T et al. Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: Correlation with clinicopathological features. Clinical Cancer Research. 1998;4(6):1527-1532

[28] Shi J, Liu Q, Sommer SS. Detection of ultrarare somatic mutation in the human TP53 gene by bidirectional pyrophosphorylation-activated polymerization allele-specific amplification. Human Mutation. 2007;28(2):131-136

[29] Taly V et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clinical Chemistry. 2013;59(12):1722-1731

[30] Newman AM et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nature Medicine. 2014;20(5):548-554

[31] Heitzer E et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Medicine. 2013;5(4):30

[32] Chan KC et al. Cancer genome scanning in plasma: Detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumor heterogeneity by massively parallel sequencing. Clinical Chemistry. 2013;59(1):211-224

[33] Song LL, Li YM. Current noninvasive tests for colorectal cancer screening: An overview of colorectal cancer screening tests. World Journal of Gastrointestinal Oncology. 2016;8(11):793-800

[34] Young GP et al. Advances in fecal occult blood tests: The FIT revolution. Digestive Diseases and Sciences. 2015;60(3):609-622
[35] Mandel JS et al. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. The New England Journal of Medicine. 1993;328(19):1365-1371

[36] Hardcastle JD et al. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. Lancet. 1996;348(9040):1472-1477

[37] Kronborg O et al. Randomised study of screening for colorectal cancer with faecal-occult-blood test. Lancet. 1996;348(9040):1467-1471

[38] Cole SR et al. A randomised trial of the impact of new faecal haemoglobin test technologies on population participation in screening for colorectal cancer. Journal of Medical Screening. 2003;10(3):117-122

[39] Lee JK et al. Accuracy of fecal immunochemical tests for colorectal cancer: Systematic review and meta-analysis. Annals of Internal Medicine. 2014;160(3):171

[40] Imperiale TF, Ransohoff DF, Itzkowitz SH. Multitarget stool DNA testing for colorectal-cancer screening. The New England Journal of Medicine. 2014;371(2):187-188

[41] Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61(5):759-767

[42] De Roock W et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: A retrospective consortium analysis. The Lancet Oncology. 2010;11(8):753-762

[43] Perrone F et al. Circulating free DNA in a screening program for early colorectal cancer detection. Tumori. 2014;100(2):115-121

[44] Dong L, Ren H. Blood-based DNA methylation biomarkers for early detection of colorectal cancer. Journal of Proteomics and Bioinformatics. 2018;11(6):120-126

[45] de Vos T et al. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. Clinical Chemistry. 2009;55(7):1337-1346

[46] Toth K et al. Free circulating DNA based colorectal cancer screening from peripheral blood: The possibility of the methylated septin 9 gene marker. Orvosi Hetilap. 2009;150(21):969-977

[47] Church TR et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut. 2014;63(2):317-325

[48] Yan S et al. Diagnostic value of methylated Septin9 for colorectal cancer screening: A meta-analysis. Medical Science Monitor. 2016;22:3409-3418

[49] Jin P et al. Performance of a second-generation methylated SEPT9 test in detecting colorectal neoplasm. Journal of Gastroenterology and Hepatology. 2015;30(5):830-833

[50] Toth K et al. Detection of methylated septin 9 in tissue and plasma of colorectal patients with neoplasia and the relationship to the amount of circulating cell-free DNA. PLoS One. 2014;9(12):e115415

[51] Pedersen SK et al. Evaluation of an assay for methylated BCAT1 and IKZF1 in plasma for detection of colorectal neoplasia. BMC Cancer. 2015;15:654

[52] Symonds EL et al. A blood test for methylated BCAT1 and IKZF1 vs. a fecal immunochemical test for detection of colorectal neoplasia. Clinical and Translational Gastroenterology. 2016;7:e137
[53] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA: A Cancer Journal for Clinicians. 2018;68(1):7-30

[54] Goldberg RM et al. Surgery for recurrent colon cancer: Strategies for identifying resectable recurrence and success rates after resection. Eastern Cooperative Oncology Group, the North Central Cancer Treatment Group, and the Southwest Oncology Group. Annals of Internal Medicine. 1998;129(1):27-35

[55] Tas F et al. Measurement of serum CA 19-9 may be more valuable than CEA in prediction of recurrence in patients with gastric cancer. American Journal of Clinical Oncology. 2001;24(2):148-149

[56] Aloe S et al. Prognostic value of serum and tumor tissue CA 72-4 content in gastric cancer. The International Journal of Biological Markers. 2003;18(1):21-27

[57] Brannon AR et al. Comparative sequencing analysis reveals high genomic concordance between matched primary and metastatic colorectal cancer lesions. Genome Biology. 2014;15(8):454

[58] Ryan BM et al. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: Strong prognostic indicator in postoperative follow up. Gut. 2003;52(1):101-108

[59] Wang JY et al. Molecular detection of APC, K-ras, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. World Journal of Surgery. 2004;28(7):721-726

[60] Lee HS et al. Circulating methylated septin 9 nucleic acid in the plasma of patients with gastrointestinal cancer in the stomach and colon. Translational Oncology. 2013;6(3):290-296

[61] Bazan V et al. Molecular detection of TP53, Ki-Ras and p16INK4A promoter methylation in plasma of patients with colorectal cancer and its association with prognosis. Results of a 3-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. Annals of Oncology. 2006;17(Suppl 7):vii84-vii90

[62] Frattini M et al. Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. Cancer Letters. 2008;263(2):170-181

[63] Tie J et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Science Translational Medicine. 2016;8(346):346ra92

[64] Young GP et al. A cross-sectional study comparing a blood test for methylated BCAT1 and IKZF1 tumor-derived DNA with CEA for detection of recurrent colorectal cancer. Cancer Medicine. 2016;5(10):2763-2772

[65] Symonds EL et al. Circulating tumour DNA for monitoring colorectal cancer—A prospective cohort study to assess relationship to tissue methylation, cancer characteristics and surgical resection. Clinical Epigenetics. 2018;10:63

[66] Tabernero J et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: A retrospective, exploratory analysis of the CORRECT trial. The Lancet Oncology. 2015;16(8):937-948

[67] El Messaoudi S et al. Circulating DNA as a strong multimarker prognostic tool for metastatic colorectal cancer patient management care. Clinical Cancer Research. 2016;22(12):3067-3077
[68] Lefebure B et al. Prognostic value of circulating mutant DNA in unresectable metastatic colorectal cancer. Annals of Surgery. 2010;251(2):275-280

[69] Bettegowda C et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science Translational Medicine. 2014;6(224):224ra24

[70] Fan G et al. Prognostic value of circulating tumor DNA in patients with colon cancer: Systematic review. PLoS One. 2017;12(2):e0171991

[71] Spindler KL et al. Changes in mutational status during third-line treatment for metastatic colorectal cancer—Results of consecutive measurement of cell free DNA, KRAS and BRAF in the plasma. International Journal of Cancer. 2014;135(9):2215-2222

[72] Goldstein MJ, Mitchell EP. Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer. Cancer Investigation. 2005;23(4):338-351

[73] Tie J et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Annals of Oncology. 2015;26(8):1715-1722

[74] Barault L et al. Discovery of methylated circulating DNA biomarkers for comprehensive non-invasive monitoring of treatment response in metastatic colorectal cancer. Gut. 2017

[75] Garcia-Saenz JA et al. Tumor burden monitoring using cell-free tumor DNA could be limited by tumor heterogeneity in advanced breast cancer and should be evaluated together with radiographic imaging. BMC Cancer. 2017;17(1):210

[76] Bernabe R et al. What do we need to make circulating tumour DNA (ctDNA) a routine diagnostic test in lung cancer? European Journal of Cancer. 2017;81:66-73

[77] Hao YX et al. Effectiveness of circulating tumor DNA for detection of KRAS gene mutations in colorectal cancer patients: A meta-analysis. OncoTargets and Therapy. 2017;10:945-953

[78] Vidal J et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Annals of Oncology. 2017;28(6):1325-1332

[79] Schmiegel W et al. Blood-based detection of RAS mutations to guide anti-EGFR therapy in colorectal cancer patients: Concordance of results from circulating tumor DNA and tissue-based RAS testing. Molecular Oncology. 2017;11(2):208-219

[80] Misale S et al. Resistance to anti-EGFR therapy in colorectal cancer: From heterogeneity to convergent evolution. Cancer Discovery. 2014;4(11):1269-1280

[81] Diaz LAJ et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486(7404):537-540

[82] Bertotti A et al. The genomic landscape of response to EGFR blockade in colorectal cancer. Nature. 2015;526(7572):263-267

[83] Siena S et al. Dynamic molecular analysis and clinical correlates of tumor evolution within a phase II trial of panitumumab-based therapy in metastatic colorectal cancer. Annals of Oncology. 2018;29(1):119-126

[84] Siravegna G et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nature Medicine. 2015;21(7):827
[85] Spindler KL et al. Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. Clinical Cancer Research. 2012;18(4):1177-1185

[86] Van Emburgh BO et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. Nature Communications. 2016;7:13665

[87] Arena S et al. Emergence of multiple EGFR extracellular mutations during Cetuximab treatment in colorectal Cancer. Clinical Cancer Research. 2015;21(9):2157-2166

[88] Mohan S et al. Changes in colorectal carcinoma genomes under anti-EGFR therapy identified by whole-genome plasma DNA sequencing. PLoS Genetics. 2014;10(3):e1004271

[89] Bardelli A et al. Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer Discovery. 2013;3(6):658-673

[90] Russo M et al. Acquired resistance to the TRK inhibitor Entrectinib in colorectal cancer. Cancer Discovery. 2016;6(1):36-44

[91] Lanman RB et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. PLoS One. 2015;10(10):e0140712

[92] Newman AM et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nature Biotechnology. 2016;34(5):547-555

[93] Sonia Mansukhani LJB, Moorcraft SY, Davidson M, Woolston A, et al. Ultra-sensitive mutation detection and genome-wide DNA copy number reconstruction by error corrected circulating tumour DNA sequencing. bioRxiv. 2017;64(11):1626-1635

[94] Lee BB et al. Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. Clinical Cancer Research. 2009;15(19):6185-6191

[95] Tanzer M et al. Performance of epigenetic markers SEPT9 and ALX4 in plasma for detection of colorectal precancerous lesions. PLoS One. 2010;5(2):e9061

[96] Cassinotti E et al. DNA methylation patterns in blood of patients with colorectal cancer and adenomatous colorectal polyps. International Journal of Cancer. 2012;131(5):1153-1157

[97] Herbst A et al. Methylation of NEUROG1 in serum is a sensitive marker for the detection of early colorectal cancer. The American Journal of Gastroenterology. 2011;106(6):1110-1118

[98] Lam K et al. DNA methylation based biomarkers in colorectal cancer: A systematic review. Biochimica et Biophysica Acta. 2016;1866(1):106-120

[99] Rasmussen SL et al. Hypermethylated DNA, a circulating biomarker for colorectal cancer detection. PLoS One. 2017;12(7):e0180809

[100] Pedersen SK et al. A two-gene blood test for methylated DNA sensitive for colorectal cancer. PLoS One. 2015;10(4):e0125041

[101] Gkountela S et al. Recent advances in the biology of human circulating tumour cells and metastasis. ESMO Open. 2016;1(4):e000078

[102] Diehl F et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proceedings of
the National Academy of Sciences of the United States of America. 2005;102(45):16368-16373

[103] Grimm M et al. High phosphorus intake only slightly affects serum minerals, urinary pyridinium crosslinks and renal function in young women. European Journal of Clinical Nutrition. 2001;55(3):153-161

[104] Peach G et al. Prognostic significance of circulating tumour cells following surgical resection of colorectal cancers: A systematic review. British Journal of Cancer. 2010;102(9):1327-1334

[105] Rahbari NN et al. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. Gastroenterology. 2010;138(5):1714-1726

[106] Yang C et al. Prognostic and clinicopathological significance of circulating tumor cells detected by RT-PCR in non-metastatic colorectal cancer: A meta-analysis and systematic review. BMC Cancer. 2017;17(1):725

[107] Kin C et al. Colorectal cancer diagnostics: Biomarkers, cell-free DNA, circulating tumor cells and defining heterogeneous populations by single-cell analysis. Expert Review of Molecular Diagnostics. 2013;13(6):581-599

[108] Simpson RJ et al. Exosomes: Proteomic insights and diagnostic potential. Expert Review of Proteomics. 2009;6(3):267-283

[109] Gold B et al. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology. The Journal of Molecular Diagnostics. 2015;17(3):209-224

[110] Valadi H et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology. 2007;9(6):654-659

[111] Uratani R et al. Diagnostic potential of cell-free and exosomal microRNAs in the identification of patients with high-risk colorectal adenomas. PLoS One. 2016;11(10):e0160722

[112] He Y et al. Current state of circulating microRNAs as cancer biomarkers. Clinical Chemistry. 2015;61(9):1138-1155

[113] Mohammadi A, Mansoori B, Baradaran B. The role of microRNAs in colorectal cancer. Biomedicine & Pharmacotherapy. 2016;84:705-713

[114] Masuda T et al. MicroRNAs as biomarkers in colorectal cancer. Cancers (Basel). 2017;9(9)

[115] Toiyama Y et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. Journal of the National Cancer Institute. 2013;105(12):849-859

[116] Yu W et al. Circulating microRNA-21 as a potential diagnostic marker for colorectal cancer: A meta-analysis. Molecular and Clinical Oncology. 2016;4(2):237-244

[117] Vychytilova-Faltejskova P et al. Serum-based microRNA signatures in early diagnosis and prognosis prediction of colon cancer. Carcinogenesis. 2016;37(10):941-950

[118] Carter JV et al. A highly predictive model for diagnosis of colorectal neoplasms using plasma microRNA: Improving specificity and sensitivity. Annals of Surgery. 2016;264(4):575-584

[119] Xie T et al. MicroRNAs as regulators, biomarkers and therapeutic targets in the drug resistance of colorectal cancer. Cellular Physiology and Biochemistry. 2016;40(1-2):62-76
[120] Ryan E et al. The current value of determining the mismatch repair status of colorectal cancer: A rationale for routine testing. Critical Reviews in Oncology/Hematology. 2017;116:38-57

[121] Buza N, Ziai J, Hui P. Mismatch repair deficiency testing in clinical practice. Expert Review of Molecular Diagnostics. 2016;16(5):591-604

[122] Le DT et al. PD-1 blockade in tumors with mismatch-repair deficiency. The New England Journal of Medicine. 2015;372(26):2509-2520

[123] Overman MJ et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): An open-label, multicentre, phase 2 study. The Lancet Oncology. 2017;18(9):1182-1191

[124] Cabel L et al. Clinical potential of circulating tumour DNA in patients receiving anticancer immunotherapy. Nature Reviews. Clinical Oncology. 2018;15(10):639-650

[125] Hause RJ et al. Classification and characterization of microsatellite instability across 18 cancer types. Nature Medicine. 2016;22(11):1342-1350

[126] Ladas I et al. Enhanced detection of microsatellite instability using pre-PCR elimination of wild-type DNA homopolymers in tissue and liquid biopsies. Nucleic Acids Research. 2018;46(12):e74

[127] Millholland JM et al. Detection of low frequency FGFR3 mutations in the urine of bladder cancer patients using next-generation deep sequencing. Research and Reports in Urology. 2012;4:33-40

[128] Fujii T et al. Mutation-enrichment next-generation sequencing for quantitative detection of KRAS mutations in urine cell-free DNA from patients with advanced cancers. Clinical Cancer Research. 2017;23(14):3657-3666

[129] Li Y et al. Salivary transcriptome diagnostics for oral cancer detection. Clinical Cancer Research. 2004;10(24):8442-8450