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Review Article

Circulating tumor DNA in colorectal cancer: opportunities and challenges

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Abstract: Tumor biopsy is the standard method for cancer diagnosis and provides an important sample for pathological assessment. With the development of precision medicine, liquid biopsies are now an important tool to detect molecular changes and tumor heterogeneity. In recent years, research related to circulating tumor DNA (ctDNA) has intensified due to its non-invasive, convenient, comprehensive, and safety characteristics. Herein, we provide a review describing the clinical applications and prospects of ctDNA in colorectal cancer (CRC) diagnosis, monitoring and prognosis.

Keywords: Liquid biopsy, circulating tumor DNA, colorectal cancer

Introduction

Colorectal cancer (CRC) is the second most common cause of cancer deaths worldwide: The mortality rate is the fourth highest among men and third highest among women [1]. The early diagnosis and treatment of CRC is necessary for clinical progress that improves patient outcomes. Importantly, early CRC detection can significantly improve the cure rate. Traditional clinical diagnostic methods include serum tumor markers, colonoscopy, imaging, and tissue biopsy. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are used as serum tumor markers, but these two markers alone do not fully satisfy clinical needs due to their lack of sensitivity and specificity [2]. Tumor biopsies also have clinical shortcomings. Due to substantial trauma and poor patient compliance, it is difficult to obtain repeat biopsies to monitor disease progression. Therefore, circulating tumor DNA (ctDNA) has emerged as a promising diagnostic tool for CRC. Furthermore, the information obtained from ctDNA and tissue biopsies are complementary. Incorporating information from ctDNA can overcome some of the challenges associated with tumor heterogeneity and limited tissue availability. This article summarizes the clinical applications and prospects of ctDNA for early detection, postoperative monitoring, treatment response and therapeutic resistance in CRC (**Figure 1**; **Table 1**).

Overview of ctDNA

Cell-free DNA (cfDNA) is fragmented DNA that is found in the non-cellular blood components of healthy individuals. Among tumor patients, ctDNA is 150~200 base pair fragments that are released by tumor cells into the bloodstream and represents a small fraction of the total cfDNA. Importantly, ctDNA retains epigenetic characteristics and carries tumor-specific mutations that can be detected in peripheral blood [3]. The normal half-life of ctDNA is less than an hour, which suggests in can reflect dynamic tumor characteristics. Studies now suggest that ctDNA has multiple origins and is not derived from a single source [4]. The three primary sources of ctDNA are: 1) apoptotic or necrotic tumor cells; 2) active tumor cells; and 3) circulating tumor cells (**Figure 2**) [5-8]. Because the genetic information carried by ctDNA is exactly the same as tumor cells and it is present in the peripheral blood, ctDNA is an ideal diagnostic tool for CRC, and its clinical applications are actively being investigated.

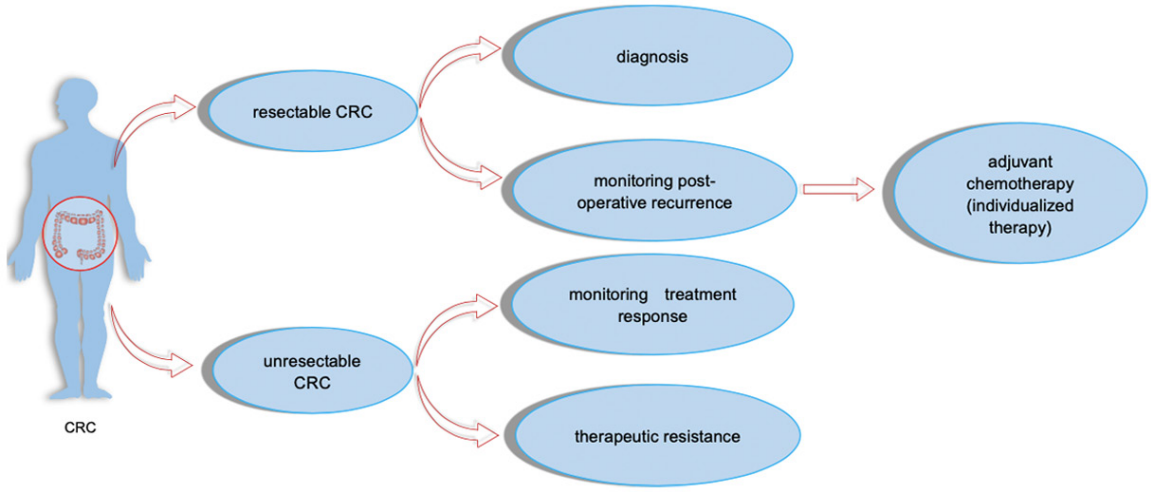

Figure 1. Clinical application of ctDNA in colorectal cancer (CRC). The primary application of ctDNA in resectable and unresectable CRC.

Extraction and detection of ctDNA

Isolating and detecting ctDNA is a significant challenge. First, ctDNA accounts for only a small portion of the total cfDNA in peripheral blood (sometimes <0.01%), which makes it difficult to obtain [9]. Most cfDNA is present in nucleosomal fragments. The ctDNA yield is usually less than 10 ng per mL of blood, with an average yield of 6.6 ng per mL. Yield can vary due to exercise, inflammation, surgery, or tissue damage [10, 11]. In clinical practice, collecting additional blood samples from patients could improve detection sensitivity, however, this is not a viable solution. Recently, enhanced detection and analysis of ctDNA with fragment size analysis drew worldwide attention. Mouliere et al. used shallow whole-genome sequencing to analyze ctDNA fragment sizes in 344 plasma samples from 200 cancer patients. The ctDNA enrichment of tumor-related fragments between 90 and 150 base pairs in length was detected. Using this method, 95% of patients had a ctDNA concentration that was more than 2 times greater than previously detected concentrations. Moreover, 10% had a concentration that was more than 4 times greater than previously obtained ctDNA concentrations [12]. This study also contributed to improvements in the ctDNA diagnostic tests with the minimal cost. Current research demonstrates that plasma is the best sample type for ctDNA analysis. Even though cfDNA concentrations are approximately 20 times higher in serum when compared to plasma, the large quantity of normal cfDNA in serum from leukocyte lysis hinders ctDNA detection [13, 14]. Therefore, blood should be collected in anticoagulant tubes containing cell stabilizers or EDTA tubes, preferably within 6 hours after sampling, to separate plasma [15].

Sanger sequencing was initially used to detect ctDNA. However, Sanger sequencing has several limitations including complicated processing and high costs [16]. Diehl et al. developed a technique called BEAMing to detect ctDNA in blood [10]. This detection technology combines digital PCR and flow cytometry. Each type of DNA molecule is specifically linked to magnetic beads, which allows for differences between DNA molecules to be evaluated by flow cytometry. Because this method is based on beads, emulsion, amplification, and magnetism, which are the four main components, it is called BEAMing. In CRC, the advent of next-generation sequencing (NGS) technology has made ctDNA detection in plasma a promising practice [17]. The sensitivity of ctDNA detection with NGS can be improved further when combined with whole genome analysis [18]. Presently, several commercially available NGS systems are suitable for clinical use including the Illumina MiseqDx, Thermo Fisher Ion Personal Genome Machine (PGM™) System, and QIAGEN GeneReader NGS System. NGS clinical use is becoming more widespread because it allows for large-scale parallel sequencing that can be performed at the whole-genome level with high

Table 1. Summary of ctDNA clinical applications

Clinical application	Analyses	Summary
Diagnosis of early stage patients	ctDNA methylation	ctDNA methylation has better sensitivity and specificity in early stage CRC patients [20-22]
	Circulating protein levels and mutations in cfDNA	ctDNA can be used to identify the site of origin for a few tumor types [29]
	Fragment length distribution of DNA types	Healthy people and cancer patients can be distinguished according to the fragment length distribution pattern of cfDNA [28]
Monitoring postoperative recurrence	ddPCR, ctDNA quantification	ctDNA can be used to predict and identify recurrence earlier [10, 33-35]
	NGS, ctDNA quantification	ctDNA can be used to stratify the risks of patients who have completed postoperative adjuvant chemotherapy and identify patients that are at high-risk for recurrence [36-38]
Monitoring treatment response	Amplicon-based deep sequencing, ctDNA quantification	ctDNA can be used to track treatment responses and inform prognoses weeks to months earlier than imaging [45, 47-50]
	ddPCR, ctDNA quantification	ctDNA levels can reflect the tumor burden in advanced patients and guide subsequent treatment [51-54]
Therapeutic resistance in metastatic patients	Real Time PCR, ctDNA mutations	ctDNA can be used to monitor acquired resistance to targeted therapy in mCRC patients and reveal resistant mechanisms in different tumor lesions within the same patient [44, 66-68, 72, 73]
	ddPCR and NGS, ctDNA mutations	ctDNA can be used to identify patients that are suitable for re-challenge strategies [69, 70]

ctDNA: circulating tumor DNA; cfDNA: cell-free DNA; CRC: colorectal cancer; mCRC: metastatic colorectal cancer; PCR: polymerase chain reaction; ddPCR: droplet digital polymerase chain reaction; NGS: next generation sequencing.

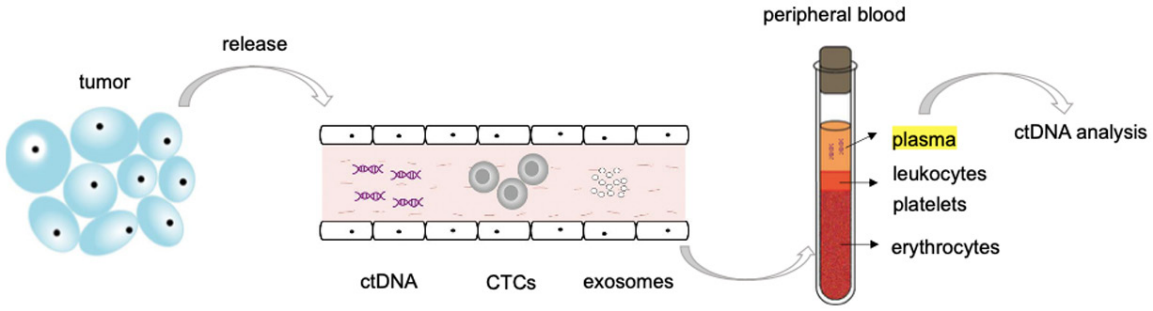

Figure 2. Overview of liquid biopsy. Liquid biopsy include circulating tumor DNA (ctDNA), which is released by tumor cells; circulating tumor cells (CTCs), which is a variety of tumor cells existing in peripheral blood; exosomes, which are extracellular vesicles released by tumor cells. The centrifuged blood sample is divided into three layers, which contains the top layer of plasma, the middle layer leukocytes and platelets, and the bottom layer of erythrocytes. Moreover, the plasma is the best sample type for ctDNA analysis.

accuracy and sensitivity. In the 2019 NCCN guidelines, NGS technology was permitted for the detection of RAS (KRAS and NRAS) and BRAF mutations in mCRC.

Clinical applications of ctDNA

Diagnosis in early-stage CRC patients

The early diagnosis of CRC is critical to improving the disease cure rate. Currently, the primary methods for early diagnosis are stool occult blood detection, digital rectal examination (DGE), and serum tumor marker (CEA, CA19-9) analysis. Detection of the serum tumor marker CEA cannot fully satisfy clinical needs due to its low sensitivity and specificity. Thus, ctDNA, especially when combined with CEA for early detection, may be an advantageous tool to diagnose CRC [19]. Some studies suggest that ctDNA methylation has better sensitivity than traditional serum tumor markers in early stage CRC patients and is a potential biomarker for CRC diagnosis [20, 21]. Moreover, a recent study indicated that a single ctDNA methylation marker, cg10673833, could yield high sensitivity (89.7%) and specificity (86.8%) for detection of CRC in 1493 participants [22]. The Epi proColon test, which is approved by the FDA for clinical application, is widely used to screen the methylation status of the SEPT9 promoter in cfDNA from CRC patients [23].

In addition to cancer, an increase in plasma ctDNA levels is observed in many other medical conditions including myocardial infarction [24], severe infection, inflammatory diseases [25], and pregnancy [26]. In the general population, benign lesions may have the same mutations as cancer cells and may release cfDNA into the circulation [27]. Therefore, an increase in ctDNA levels may be non-specific. To overcome this problem, a recent study found that DNA fragment length distributions exhibit distinct patterns. The length distribution of cfDNA fragments from healthy individuals was regular. In contrast, the length distribution of ctDNA fragments from cancer patients was irregular [28]. These data indicated that healthy individuals and cancer patients can be distinguished according to the distribution patterns of free DNA fragment lengths. Other studies have demonstrated conserved gene mutations among tumors, including those in KRAS, BRAF, and TP53. Unfortunately, it is difficult to locate minimal tumors in specific organs when a test shows a positive result. In this scenario, a test called CancerSEEK can be performed to evaluate circulating protein levels and cfDNA mutations. The sensitivity of CancerSEEK in five cancer types (ovary, liver, stomach, pancreas, and esophagus) was 69-98%, and the specificity was greater than 99% in all tumor types. These results provide evidence that CancerSEEK improves early stage cancer detection. Most importantly, CancerSEEK identifies the site of origin for select tumor types [29].

It has been shown that ctDNA primarily originates from necrotic and apoptotic tumor cells, and it is necessary to quantify the effective level of cfDNA in healthy individuals to create better diagnostic thresholds for early diagnosis [30]. Therefore, the ctDNA field needs support from studies with large sample sizes, and the detection methods must be standardized be-

fore they can be used for clinical cancer diagnoses.

Monitoring postoperative early recurrence in CRC

During the clinical treatment of CRC, most patients with stage I-III non-metastatic CRC receive radical surgery. Among these patients, 30-50% still face disease recurrence [31, 32]. Therefore, it is necessary to identify an effective biomarker to monitor disease relapse. Current monitoring methods, including serum tumor marker, imaging, and colonoscopy, cannot identify early relapse. Preliminary research indicates that ctDNA levels are notably decreased in patients without disease recurrence and elevated in patients with recurrence [10, 33, 34]. Compared with the traditional tools for monitoring disease relapse, ctDNA can identify a recurrence 2-15 months (average 10 months) earlier [35]. This is a major breakthrough for clinical practice.

At the 2019 American Society of Clinical Oncologists (ASCO) conference, Tie et al. reported the results of a prospective study that included 485 CRC patients with stage II or III disease. The study suggested that the prognostic significance of ctDNA for postoperative adjuvant chemotherapy can be further stratified by ctDNA minor-allele frequency (MAF) level. This study reported that the 3-year relapse free survival (RFS) for patients with MAF>0.046% was 9% and was 33% for patients with MAF≤0.046%. For patients receiving adjuvant chemotherapy, the 3-year RFS for MAF>0.046% was 25%, compared to 70% for MAF≤0.046% [36]. For patients with stage III colon cancer, National Comprehensive Cancer Network (NCCN) guidelines recommend postoperative adjuvant chemotherapy to clear minimal residual disease. However, it is still unclear who has a high risk of recurrence after adjuvant chemotherapy. Recent studies suggest that ctDNA can stratify the risks of patients who have completed adjuvant chemotherapy to identify high-recurrence risk groups [37]. At the 2019 European Society for Medical Oncology (ESMO) conference, the IDEA-France Phase III clinical trial stimulated important conversation. ctDNA can guide individualized adjuvant chemotherapy therapy in high risk patients. For patients with low risk CRC (T3N1), positive ctDNA levels after surgical resection, especially after 3 months of chemotherapy, indicate that treatment should be extended to 6 months. As Dasari et al. reported in a recent editorial, ctDNA provides new therapeutic opportunities for patients at high-risk for recurrence [38]. Of course, the current problem is whether patients with a high recurrence risk confirmed by ctDNA should receive intensive treatment. All of these issues need a large number of prospective studies to obtain answers.

Wang et al. also proposed that monitoring ctDNA levels every 3-6 months after surgery can be used to supplement CEA, CT, or other conventional monitoring tools. It also can be used to stratify CRC patients after surgical resection [39]. The study also emphasized that even though the average time before a ctDNA positive test was 4 months prior to imaging detection, the ctDNA result still appeared 9 months after surgical resection. Overall, ctDNA detection did not affect the decision to pursue adjuvant chemotherapy in clinical practice. There are some lingering questions about how to make clinical decisions when ctDNA indicates a possible recurrence, but imaging does not provide an obvious confirmation during a follow-up, and how to choose the appropriate treatment strategies. The answers to these questions lack clinical results, and more prospective studies are needed to guide the treatment of patients with postoperative recurrence.

The detection of ctDNA can identify minimal residual disease and characterize the risk stratification of adjuvant chemotherapy. It can effectively manage postoperative treatment in CRC patients. In the future, it is anticipated that data from prospective studies will allow ctDNA to be used for clinical monitoring of CRC recurrences [39, 40]. Therefore, the application of ctDNA to postoperative monitoring fully reflects the concept of individualized precision medicine and is superior to traditional monitoring methods because it can identify recurrences earlier.

Monitoring treatment response in CRC

Traditionally, to monitor advanced CRC, CEA, imaging (CT, MRI), and colonoscopy are used for evaluation. However, CEA has significant limitations in clinical practice due to its low sensitivity and specificity [41-43]. Consequently,

better biomarkers are needed to monitor treatment response and ctDNA is a suitable alternative [44]. As already discussed, the tumor burden can be monitored in real-time due to the short half-life of ctDNA. Compared to imaging approaches, serial ctDNA analysis can be used to track treatment responses weeks to months earlier, which can provide sufficient time to adjust treatment strategies and prevent disease progression [45, 46]. The plasma ctDNA level is related to poor prognosis in the patients after chemotherapy [47-49]. More specifically, Tie and colleagues reported that patients with ctDNA levels that decreased ≥10-fold after the chemotherapy had longer progression-free survival (PFS) than patients with ctDNA levels that decreased <10-fold (median PFS: 14.7 months VS 8.1 months; HR=1.87; 95% CI 0.62-5.61) [50].

In addition to identifying individuals that do not respond to treatment, ctDNA can also be used to assess the tumor burden in advanced patients and to guide subsequent treatment decisions. Vidal et al. noted that dynamic changes in RAS ctDNA MAF can reflect disease progression and that these changes are measurable before diagnostic imaging can occur [51]. A recent retrospective study confirmed the utility of ctDNA as a prognostic biomarker for metastatic colorectal cancer (mCRC) before first-line oxaliplatin-based chemotherapy [52]. A prospective phase II clinical trial (NCT01442935) also indicated the clinical utility of ctDNA. For patients with liver metastases from potentially resectable CRC, receiving first-line standard chemotherapy combined with targeted therapy, which can detect ctDNA level before R0 or R1 surgery have shorter overall survival (OS) ($p<0.001$). This result also proved that ctDNA can be used to identify patients that are suitable for resection of liver metastases [53]. Apatinib monotherapy efficacy in patients with refractory mCRC was also evaluated with ctDNA [54]. The ctDNA levels in the peripheral blood of patients with mCRC was higher than the ctDNA levels in patients with non-metastatic CRC. It is clinically significant to monitor the treatment response in patients with advanced CRC [55]. As an important tool, ctDNA can inform better treatment strategies for advanced disease patients and has a greater potential to supplement Response Evaluation Criteria in Solid Tumors (RECIST) evaluation.

Neoadjuvant chemoradiotherapy (nCRT) is widely used to treat locally advanced rectal cancer (LARC). Approximately 50-60% of rectal cancer patients suffer from tumor regression after neoadjuvant chemoradiotherapy, with a pCR (pathologic complete response) as high as 20% [56]. To avoid postoperative complications and improve the quality of life in advanced LARC patients, some researchers have proposed a “watch and wait” strategy. Previous studies confirm that the response of rectal cancer patients to neoadjuvant therapy is related to the disease prognosis [57]. Yang et al. suggested that ctDNA can be used to classify patients with LARC into high-risk or low-risk subgroups and therefore, choose patients that are suitable for a “watch and wait” strategy [58]. Although ctDNA can provide valuable information to inform treatment decisions, prospective research is still needed to evaluate follow-up treatment strategies.

In clinical practice, ctDNA has great potential for improved treatment monitoring. It avoids radiation exposure from conventional CT scans and has higher sensitivity than CEA. Therefore, ctDNA can be used for better identification of disease progression and to make timely adjustments to treatment strategies.

Therapeutic resistance in metastatic patients

The mCRC patient subpopulation with wild-type KRAS/NRAS/BRAF is usually sensitive to initial anti-EGFR therapy. However, tumors commonly develop acquired resistance within the first few months of treatment, which is the main cause of treatment failure for individuals receiving targeted tumor therapy. The mechanism of acquired resistance during anti-EGFR therapy is attributed to bypass signal pathway activation and secondary alterations in the EGFR receptor [59-61]. Approximately 40% of CRC patients have mutations in codons 12 and 13 of the KRAS exon 2 [62]. Some studies have shown that mutations in the KRAS exon 2 predict anti-EGFR (cetuximab and panitumumab) treatment failure [63-65]. The mechanism of acquired resistance is complicated in anti-EGFR therapy and is difficult to track in clinical practice. However, because ctDNA can be used for non-invasive, real-time monitoring of abnormalities in the EGFR signaling pathway, it could be a strategy to identify acquired resistance to

anti-EGFR therapy in mCRC patients and guide subsequent treatment.

Retrospective analysis indicates that KRAS mutations can develop in patients who were initially KRAS wild-type while receiving anti-EGFR therapy. Accordingly, other studies have found that KRAS mutations appear within 10 months of cetuximab therapies. These results indicate that KRAS mutations are a primary mechanism of acquired resistance with anti-EGFR treatment and usually develop within 5-6 months of treatment [44, 66]. Because different resistant mechanisms will occur in different tumor lesions within the same patient [67], ctDNA can be a powerful tool. Parallel analysis of serial ctDNA monitoring can non-invasively track these mutations to reduce the adverse effects caused by tissue biopsies and to guide follow-up treatments [68]. Currently, anti-EGFR re-challenge strategies are effective in patients with acquired resistance [69]. For example, cetuximab combined with irinotecan is effective as a third-line therapy for patients that received cetuximab combined with irinotecan as a first-line therapy prior to developing resistance. Patients that are suitable for a re-challenge strategy can be selected by analyzing ctDNA [70].

HER2 belongs to the same family of signaling kinase receptors as EGFR. Notably, successful targeting of HER2 in breast cancer patients has been achieved in both the advanced and adjuvant settings. HER2 overexpression in colorectal cancer is rare and only occurs in approximately 3% of patents [71]. Intriguingly, the HER2 overexpression rate in patients with wild-type KRAS/NRAS/BRAF is approximately 5-14% [71]. HER2 overexpression also indicates failure of anti-EGFR therapy. A study showed that HER2 overexpression can be non-invasively detected with ctDNA and used to predict the efficacy of anti-HER2 targeted therapy [72].

In current immunotherapy, tumors with microsatellite instability-high (MSI-H) status are sensitive to immune checkpoint inhibitors (ICBs), but nearly half of the patients in this subpopulation have innate resistance. Activation of the WNT/β-catenin pathway can lead to immunological rejection and resistance to ICBs. Recent studies indicate that patients with MSI-H who are also resistant to ICB have an RNF43 mutation and additional mutations in APC or CTNNB1. These studies also indicated that co-activation the of WNT/β-catenin pathway promotes resistance mechanisms [73]. The analysis of ctDNA to identify resistance mechanisms has highlighted the clinical potential of liquid biopsies. Serial ctDNA analysis can identify secondary resistance mechanisms that are not captured by single tissue biopsy while simultaneously predicting the time and reason for treatment failure. These analyses can play a key role in guiding clinical therapeutic strategies.

Prospective outlook of ctDNA applications in CRC

As a potential tool for clinical practice, ctDNA has a promising future. However, there are still several areas of the liquid biopsy technology that require development including the clinical examination method, a standardized detection process, and quantitative standards. Variables that affect the sample quality, including sample collection, transportation, and storage, should be controlled. In addition, it is still difficult to separate specific ctDNA fragments from cfDNA. Selecting the best detection panel is also an ongoing challenge. Although ctDNA fragments are currently enriched based on ctDNA/cfDNA fragment length, more research in this area is needed to perform this is the best practice. Currently, the utility of ctDNA is not limited to quantitative assessment, but also provides information related to mutations, copy number variation, and epigenetics. A large quantity of prospective studies with ctDNA are still needed to prove its clinical utility. There are some lingering questions about how to make clinical decisions when ctDNA indicates a possible recurrence, but imaging does not provide an obvious confirmation during a follow-up, and the patients with ctDNA-positive whether need intensive therapy. Needless to say, key benefits of ctDNA are that it provides better metrics for precision medicine and that it breaks away from the limitations of tumor tissue biopsies. Furthermore, ctDNA enables non-invasive treatment monitoring and can inform prognostic evaluations. Ongoing prospective clinical trials with ctDNA are focused on the diagnosis, surveillance, and prognosis of CRC. With the rapid development of science and technology, liquid biopsies will certainly play a key role in the diagnosis and treatment of CRC.

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Disclosure of conflict of interest

None.

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