Circulating tumor DNA detection: A potential tool for colorectal cancer management (Review)

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Abstract. Colorectal cancer (CRC) is frequently diagnosed at an advanced stage of the disease, the pathogenesis of which is influenced by genetic and epigenetic events. Circulating tumor DNA (ctDNA) is extracellular DNA that is present in a number of bodily fluids, including blood, synovial fluid and cerebrospinal fluid. Compared with performing a tissue biopsy, ctDNA examination presents the advantages of minimal invasion and greater convenience. ctDNA is commonly used to identify actionable genomic alterations, monitor treatment responses, unravel therapeutic resistance and potentially detect disease progression prior to clinical and radiological confirmation. The technique can potentially serve as a non-invasive diagnostic tool in personalized medicine, as it demonstrates prognostic value in the management of patients with CRC. ctDNA detection continues to demonstrate inherent advantages compared with other methods, thus serving an increasingly important role in tumor monitoring and oncotherapy. The aim of the current review was to explore the clinical applications of ctDNA in patients with CRC, including early detection and screening, medication guidance, resistance prediction, and residual lesion and recurrence monitoring. Furthermore, several technical methods for ctDNA detection and analysis are explored, as well as other potential biomarkers.

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1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-associated mortalities worldwide (1). Therefore, effective monitoring and therapy are particularly important in the daily management of the disease.

Circulating tumor DNA (ctDNA) is extracellular DNA from cancer cells that have undergone cell death, which is present in a number of bodily fluids, including blood, synovial fluid and cerebrospinal fluid; it is composed of single- or double-stranded DNA (2) (Fig. 1). ctDNA, as the first choice for liquid biopsy, has gradually evolved from research to clinical use, as its testing is non-invasive and reproducible (3), thus, it is a potential tool for detecting gene mutations.

CRC is a highly heterogeneous and complex disease involving various genotypes or subtypes of cells in tumors of the same histological type (4). As such, a single biopsy cannot fully demonstrate the complexity of the intra- and inter-tumor genome landscape. ctDNA presents a number of advantages over tissue biopsy in this aspect.

Traditional oncotherapy includes surgery, radiotherapy and chemotherapy. As each patient with CRC has a unique genetic and epigenetic background, patients with the same clinical and pathological characteristics of tumors may have very different therapy responses and survival rates. This phenomenon has considerably motivated research on personalized treatment. ctDNA may serve as a non-invasive diagnostic tool for individualized medicine, as it provides molecular information similar to that of invasive tumor biopsies (5).

2. Methods for ctDNA detection and analysis

Droplet digital polymerase chain reaction (ddPCR). ddPCR is one of the most accurate and applicable tools currently

available for examining genetic alterations (6). ddPCR is used to detect rare mutations, quantify copy number variations and evaluate microRNA, and the technique can be extended to clinical applications due to its relatively simple workflow. However, it can only be used to screen for known mutations.

Beads, emulsion, amplification and magnetics (BEAMing). BEAMing is relatively sensitive and inexpensive when assessing a limited number of potential mutations (7). However, although it is fairly accurate, similar to ddPCR, BEAMing can only screen for known mutations. Furthermore, due to the complex workflow, dedicated instrumentation and the high cost of each sample, implementation of this technique in routine clinical settings is limited. Using BEAMing may be effective for detecting RAS mutations in blood (8).

Tagged-amplicon deep sequencing (TAm-Seq). The main features of TAm-Seq include a high sequencing flux, reduced sequencing time and cost, and the ability to simultaneously sequence millions of DNA molecules, thereby enabling the analysis of the transcripts and genomes of a species in detail. Forshew *et al* (9) used this method to investigate the tumor protein p53 gene in patients with advanced ovarian cancer and proved the presence of metastatic mutations in multiple primary tumors; in this study, the detection rate of TAm-Seq was >2%, and its sensitivity and specificity were ~97%.

Cancer personalized profiling by deep sequencing (CAPP-Seq). CAPP-Seq can identify multiple mutations in patients with the same type of cancer and improve the assessment of tumor heterogeneity, thereby providing more comprehensive diagnostic information. Newman *et al* (10) detected ctDNA by CAPP-Seq in 50% of a patient population with stage I non-small cell lung cancer and 100% of patients with the same type of cancer at stages II-IV. The study identified that CAPP-Seq could detect tumor burdens prior to medical imaging, thus indicating that this technique is valuable in monitoring residual disease. CAPP-Seq can detect all major mutation types, including single nucleotide variants, insertions, rearrangements and copy number changes (11).

Whole genome sequencing (WGS). Complete rare tumor genome characterization shows great potential in assisting clinical decision-making and identifying unreserved treatment regimens, rare mutations and invisible oncogenes. However, several challenges limit the application of WGS in the clinical setting, including quality assurance, ethical issues and a lack of trained clinicians; it is also costly and time-consuming (12). Kim *et al* (13) performed WGS in ~250 untreated and 20 recurrent glioblastoma samples, and discovered that a change in the p53 pathway is a major molecular predictor of malignant glioma mutation. The results of the study thus suggested that the genetic changes in primary tumors may affect the subsequent evolution of tumor cells and the emergence of subclonal heterogeneity.

Whole exome sequencing (WES). WES can be used to sequence the coding region of the human genome, in order to detect common or rare disease-related abnormalities. Lecomte *et al* (14) used WES to determine familial pancreatic cancer, and detected partner and localizer of BRCA2 as a susceptibility gene. The results of this study indicated that WES shows promising clinical utility in locating potential oncogenes and tumor suppressor genes. For example, it may provide a reference for the molecular diagnosis of tumors. Compared with traditional sequencing technology, its low cost and high yield are major advantages. However, WES currently remains in the early stages of development.

Whole genome bisulfite sequencing (WGBS-Seq). WGBS-Seq offers single cytosine measurement resolution and high accuracy. Due to these advantages, the technique has become the gold standard in DNA methylation analysis (15). WGBS-Seq has made important contributions to the discovery of partially methylated domains in cancer cells (16). However, the method presents limitations in its operation. In particular, DNA may exist at varying degrees of degradation and the method may exhibit reduced sensitivity during detection. Table I lists the advantages and disadvantages of the various detection methods.

3. Clinical applications of ctDNA in CRC

Early detection and screening. An *in vitro* study revealed that live tumor cells could release DNA continuously, thus illustrating the feasibility of ctDNA in early-stage cancer (17). Schmiegel *et al* (18) recently reported that the RAS mutation status in plasma and tissue is consistent in patients with CRC. Underhill *et al* (19) demonstrated that the integrity of ctDNA could indicate cancer cell death, allowing the use of ctDNA fragment length as a reliable early detection marker. However, other colorectal diseases exhibit similar integrity, and whether this level of integrity could predict early cancer remains unknown. Therefore, further studies on this technique are required. The level of ctDNA in peripheral blood is closely associated with tumor initiation and progression, and patients at later stages of the disease are considered to carry more fragments than those at earlier stages (14). Furthermore, the release rate of ctDNA is associated with the location, size and vascular distribution of tumors (20). Blood tests for extensive population screening require strict specificity. Gao *et al* (21) indicated that ctDNA is not superior to protein biomarkers (carcinoembryonic antigen, CEA) in terms of sensitivity and specificity. As the most widely used serum markers in CRC, CEA presents no specific diagnostic value, as false-positive and false-negative results may affect early diagnosis. Therefore, using a combination of ctDNA and CEA may be a useful method for the diagnosis of early-stage CRC.

Medication guidance and resistance prediction. Anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, cetuximab and panitumumab, are effective treatments for advanced CRC. These therapies function by directly blocking the EGFR pathway and enhancing the activity of chemotherapy drugs (22). The National Comprehensive Cancer Network (NCCN) 2016 guidelines state that the Kirsten rat sarcoma virus oncogene homolog (KRAS) sequence must be tested prior to administering cetuximab in CRC treatment, and only patients with KRAS wild-type tumors respond with clinical efficacy. The NCCN and the European Society for

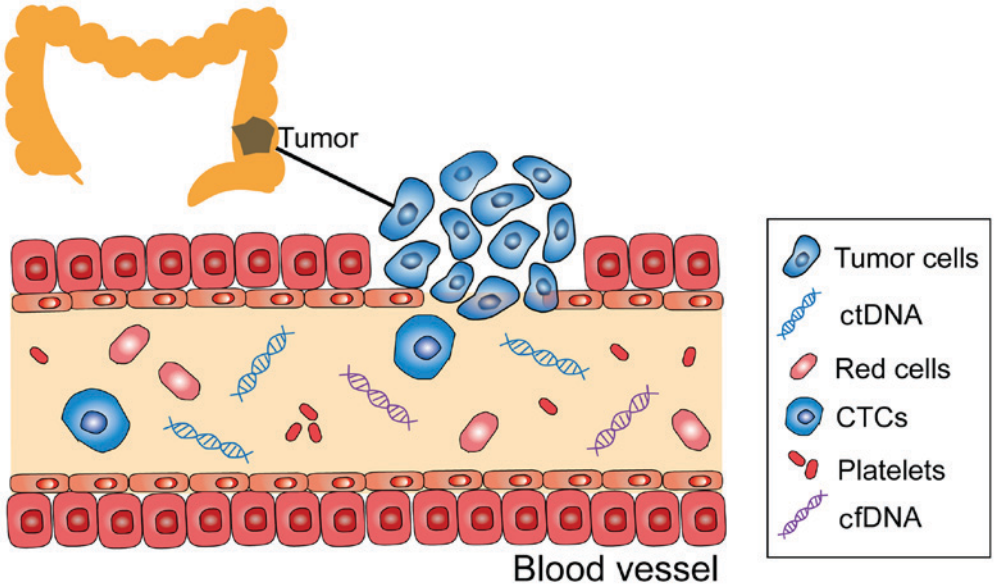

Figure 1. CTCs, ctDNA and cfDNA in the peripheral blood stream. ctDNA, circulating tumor DNA; CTC, circulating tumor cell; cfDNA, cell-free DNA.

Medical Oncology suggest avoiding cetuximab and panitumumab in patients with B-raf proto-oncogene (BRAF) mutant cancer. However, even patients with KRAS wild-type tumors could have no response to anti-EGFR therapy, particularly in the event of BRAF and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit α mutations, the overexpression of human epidermal growth factor receptor 2, MET proto-oncogene and KRAS, and the absence of phosphatase and tensin homolog expression. Tumor heterogeneity may be one of the most important reasons for the observed resistance (23). Relying on tissue biopsy to monitor resistance is unrealistic. These issues indicate the important role of ctDNA in assessing the genomic and subclone mutations repeatedly during treatment. ctDNA could also be used to monitor the clonal evolution and drug resistance of CRC in patients. ctDNA analysis could identify a second resistance mechanism not captured by single lesion biopsy (24), predicting the timing and causes of the treatment failure. Monitoring KRAS mutations in ctDNA can provide clinical guidance for the determination of treatment for patients with CRC (25). These findings indicate that ctDNA has great potential for acquired resistance monitoring.

Residual lesion and recurrence monitoring. Patients with locally advanced rectal cancer generally receive neoadjuvant chemotherapy followed by radical surgery. Sensitive monitoring of neoadjuvant chemoradiotherapy (nCRT) or post-surgical recurrence is important for patients with CRC, as early detection of recurrence is associated with increased survival times (26). Early-stage recurrence is mainly due to incomplete resectional treatments or the existence of unknown metastasis (27). A relapse rate of 50-60% was previously observed in patients with stage III cancer following complete resectional treatments (28). In general, ~80% of the recurrence occurred in the first 2 years after surgery (29,30). Carpinetti *et al* (31) compared ctDNA levels with clinical, radiological and pathological responses to nCRT, and identified that ctDNA could be used to detect disease recurrence and monitor treatment responses to nCRT, preceding an increase in CEA levels and radiological diagnosis. Diehl *et al* (32) detected independent gene mutations in patients with CRC and identified 16 cases of postoperative ctDNA in patients with recurrence and 4 cases of non-detectable ctDNA in patients with no lesions. A previous study revealed that, compared with conventional follow-up, ctDNA monitoring could identify the recurrence of CRC progression 10 months earlier than radiological reports (29), as tumor progression is accompanied by an accumulation of mutations (27). Tie *et al* (33) performed large-scale assays to determine the feasibility of using ctDNA in detecting residual disease among patients who had not been treated with adjuvant chemotherapy; ctDNA was detected postoperatively in 14 (7.9%) of 178 patients, 11 (79%) of whom exhibited recurrence at a median follow-up time of 27 months, whereas recurrence occurred in only 16 (9.8%) of the remaining 164 patients with negative ctDNA. While these findings suggest that ctDNA detection may provide direct evidence of residual disease, Habr-Gama *et al* (26) came to contrasting conclusions and suggested that whether ctDNA levels are proportional to systemic tumor burden is not clear since no independent method is yet available to detect this burden. Clinical applications of ctDNA detection in patients with CRC are summarized in Table II. Besides CRC, ctDNA detection is widely used in the detection of numerous other types of cancer (Table III). Although ctDNA has great prospects in monitoring the responses of patients post-surgery, a number of obstacles remain, including early risk prediction, real-time tracking of tumor progression and mutation detection of treatment resistance (34).

ctDNA and DNA methylation (DNAme). DNAme is an important epigenetic modification that refers to a reversible and heritable approach for regulating genomic functions; it mainly occurs in 5'-cytosine-guanine-3' (CpG) dinucleotide sites in the G/C nucleotide-intensive sequences (CpG islands) (35). With further understanding of DNA methylation mechanisms and advances in methylation chip technology, DNAme has demonstrated great value in early tumor screening, prognostic

Table I. Comparison of ctDNA detection methods.

Method	Details	Detection limit, %	Target mutation	Advantage(s)	Limitation(s)	(Refs.)
ddPCR	Absolute quantification of the initial sample	0.01-0.10	Known only	High sensitivity	Only able to detect limited genomic positions in a sample	(52-54)
BEAMing	Bead, emulsion, amplification and magnetics	0.01	Known only	High sensitivity, relatively inexpensive	Only can detect known mutations	(55,56)
TAm-Seq	First sequencing method adapted to detect rare diagnosis mutations in cell-free DNA	2.00	Known and new	High sensitivity, relatively inexpensive	Less comprehensive	(9)
CAPP-Seq	Targeted hybrid capture	0.01	Known and new	High sensitivity	Less comprehensive	(10,57,58)
WGS	Deep sequencing of entire genome	1.00	Unknown	Interrogating entire genome	Low sensitivity, expensive	(59-61)
WES	Deep sequencing of exome		Unknown	Interrogating entire exome	Low sensitivity, expensive	(59,62)
WGBS-Seq	A gold standard in DNA methylation analysis		Unknown	High accuracy and single cytosine measurement resolution	Expensive	(15)

ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction; BEAMing, bead, emulsion, amplification and magnetics; TAm-Seq, tagged-amplicon deep sequencing; CAPP-Seq, cancer personalized profiling by deep sequencing; WGS, whole genome sequencing; WES, whole exome sequencing; WGBS-Seq, whole genome bisulfite sequencing.

evaluation and chemosensitivity prediction (36-38). Detection of tumor-specific DNAme alterations in ctDNA may assist in monitoring the tumor burden and treatment responses of patients with breast or hepatocellular cancer (39). Differentially methylated regions of ctDNA were tested in healthy subjects and patients with colon cancer, and it was demonstrated that the DNAme detection method exhibits high sensitivity and specificity for CRC (40).

4. Other potential predictive biomarkers

Circulating tumor cells (CTCs). CTCs refer to various types of tumor cells with the antigenicity and genetic characteristics of a specific tumor type, found in the peripheral blood (41). The CellSearch® CTC test (Menarini Silicon Biosystems, Inc., Huntington Valley, PA, USA) is the only Food and Drug Administration-approved *in vitro* CTC diagnostic tool with positive counts associated with overall survival (OS) and progression-free survival (PFS) in patients with CRC (42). Lu *et al* (43) revealed that patients with detectable CTCs following adjuvant chemotherapy have higher recurrence rates than those without. Krebs *et al* (44) also suggested that patients with elevated CTC blood levels (≥3 cells per 7.5 ml) are more likely to benefit from intensive chemotherapy regimens than those with lower CTC levels, indicating that CTC levels may aid in guiding the adjustment of the treatment protocols of patients with CRC. In addition, CTCs are advantageous in other areas, for example, while complex chromosomal rearrangements, including translocation, cannot be easily detected using ctDNA, it is possible with CTC detection (45). The genetic and phenotypic profiles of CTCs are usually different from those of primary tumors; therefore, these biomarkers may be used to determine the most effective targeted therapy.

Cell-free DNA (cfDNA). Patients with CRC have higher total cfDNA levels (which include ctDNA) than healthy individuals (46,47), implying that cfDNA levels may distinguish patients with cancer from those without. The level of degradation of cfDNA may be a sensitive indicator of therapeutic effect and disease progression (48,49), similar to the ctDNA. Lin *et al* (50) confirmed that patients with lower cfDNA levels have improved 5-year OS rates compared with those with higher levels. Kitahara *et al* (51) evaluated the levels and integrity (as determined by the ratio of long/short fragments) of cfDNA in plasma samples collected from 93 patients with CRC prior to immunotherapy and drew similar conclusions that cfDNA integrity could be a predictive biomarker of immunotherapy efficacy. Higher cfDNA integrity tends to indicate excessive tumor necrosis factors and a high likelihood of tumor rupture; it also predicts immunosuppressive resistance and shorter PFS time in patients with CRC (49).

Table II. Clinical applications of ctDNA detection in patients with colorectal cancer.

Author, year	Patients, n	Potential clinical utility	Biomarker(s)	Detection method(s)	(Refs.)
Church *et al*, 2014	7,941	Screening	Methylated SEPT9	qPCR	(63)
Diehl *et al*, 2008	18	Prognosis	APC, KRAS, PIK3CA, TP53	BEAMing, qPCR	(32)
Cabel *et al*, 2017	15	Immunotherapy monitoring	KRAS, BRAF, EGFR, TP53	bi-PAP, NGS, ddPCR	(64)
Xu *et al*, 2017	32	Resistance	PIK3CA, KRAS, AKT1	Sanger sequencing	(65)
Vidal *et al*, 2017	115	Diagnosis and treatment monitoring	KRAS, NRAS	BEAMing	(66)
Grasselli *et al*, 2017	146	Treatment selection	KRAS, NRAS	BEAMing, qPCR	(67)
Kakizawa *et al*, 2017	16	Treatment monitoring	KRAS	ddPCR	(68)
Nq *et al*, 2017	44	Early detection of metastasis	KRAS	Multiplex-PCR amplicon sequencing	(69)
Garrigou *et al*, 2016	240	Monitoring mutations	MettDNA (WIF1, NPY, PENK)	ddPCR	(70)
Siravegna *et al*, 2015	100	Monitoring clonal evolution and resistance	KRAS, MAP2K1, NRAS, MET, FLT3, ERBB2, EGFR	BEAMing, ddPCR, qPCR	(71)
Mouliere *et al*, 2011	12	Detection of tumor weight	KRAR, ACTB	qPCR	(72)

qPCR, quantitative polymerase chain reaction; BEAMing, bead, emulsion, amplification and magnetics; bi-PAP, bidirectional pyrophosphorolysis-activated polymerization; NGS, next generation sequencing; ddPCR, droplet digital polymerase chain reaction.

Table III. Use of circulating tumor DNA detection in various cancer types.

Cancer	Diagnostic biomarker(s)	Sample size, n	Detection method(s)	(Refs.)
Head and neck squamous cell carcinoma	TP53	6	ddPCR	(73)
Non-small cell lung cancer	EGFR, T790M	10	ddPCR	(74)
	TP53, KRAS, EGFR	68	NGS	(75)
	EGFR, KRAS, PIK3CA, TP53	58	Targeted sequencing	(76)
Renal cell carcinoma	TP53, VHL, EGFR, NF1, ARID1A	220	NGS	(77)
	MET		Immunohistochemistry, direct DNA sequencing, qPCR	(78)
Breast cancer	TP53	46	ddPCR	(79)
	TP53	40	Microfluidic digital PCR	(80)
	ESR1, ERBB2, PIK3CA, AKT1 E17K	96	mdPCR	(81)

ddPCR, droplet digital polymerase chain reaction; NGS, next generation sequencing; mdPCR, multiplex digital PCR.

5. Conclusions and perspectives

With the development of novel molecular targeted agents and the application of individualized treatment, the survival rates of patients with metastatic CRC have significantly improved. There is a close association between targeted therapy and gene detection. Compared with tissue biopsy, ctDNA examination presents the advantages of minimal invasion, convenience and no contact with radioactive material. The analysis of ctDNA can therefore be used to guide immediate management, clarify drug resistance mechanisms and detect minimal residual diseases or recurrence prior to imaging. Tissue biopsy is a powerful method to obtain static information on the cancer, while ctDNA detection is more timely and accurate. The main advantage of ctDNA analysis is its high specificity, as mutations in ctDNA are consistent with those in the tumor DNA. Although ctDNA has great prospects in monitoring the responses of patients post-surgery, a number of obstacles, including early risk prediction, real-time tracking of tumor progression and mutation detection of treatment resistance, have been encountered (34). Additionally, operating procedures during the extraction of cfDNA have not been standardized, with the cost and practicality of the associated technical

methods being important issues hindering its wider clinical applications. Despite these shortcomings, ctDNA detection presents inherent advantages over other methods, indicating that it may serve an increasingly important role in tumor monitoring and oncotherapy. As the technique is gradually adopted in clinical applications, a standardized known-marker detection database could be established and a standard list of ctDNA markers across different tumor types could be made available. In addition, more detection kits accompanying diagnostic reagent standards should continue to be issued. In the future, the combination of ctDNA analysis and clinical immunotherapy could be attempted.

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Availability of data and materials

Not applicable.

Authors' contributions

HL and CJ carried out the collection and reading of the reference, and HL participated in writing of the manuscript. JW, JN, HS and XX participated in the analysis and interpretation of the references. YD, RL, SD and JF participated in the study design of the review. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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