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Review Article

How ctDNA Changing the Landscape of Management of Colorectal Cancers

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ABSTRACT

Colorectal cancer (CRC) continues to be one of the common causes of cancer-related deaths. A significant proportion of surgically cured CRC patients' relapse and if these recurrences are not resectable, they carry a poor prognosis. Despite advances in cancer treatment with targeted monoclonal antibodies, only minority benefit. At present, tissue biopsy, imaging and serum tumour markers are standard of care in the management. There is a constant need for novel biomarkers to improve risk stratification, pickup recurrences early and selection of therapy. Cellular contents of cancer cells, including their DNA, are continuously shed into circulation. This non-invasive blood-based genotyping of circulating tumour DNA (ctDNA) appears to provide genetic information similar to tumour tissue molecular profiling. Besides, ctDNA can be quantified and levels may be used to assess tumour burden. Here, we review several emerging clinical applications of ctDNA in parallel to the standard of care to improve the management of CRC.

Keywords: ctDNA, Colorectal cancers, Liquid biopsy

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths, accounting for about 1 million deaths in 2018.[1] Fortunately, the mortality of CRC is declining in developed nations.[2] The decline in mortality may be due to improvement in CRC screening, management and early detection of recurrence.[3-5] Even the median survival of metastatic CRC has improved due to the increasing number of therapeutic drugs and biomarker-driven treatment selection.[6] Molecular characterisation of the tumour helps in predicting early recurrences, disease progression and outcomes.[6,7] However, tumour tissue genotyping has many hurdles and drawbacks like an insufficient sample, need for an invasive procedure and its complications (particularly if repeat/serial biopsies are required), sampling bias and tumour heterogenicity.[8-11] To address these issues, a new diagnostic modality 'liquid biopsy' is emerging and gaining popularity in oncological care.[12,13] Liquid biopsy is a minimally invasive blood test that includes analysis of circulating tumour cells or circulating tumour DNA (ctDNA) released from tumour tissue to characterise the cancer genome.[14] In this review, we will discuss the available evidence for the clinical utility of ctDNA to improve the management and outcomes of CRC patients.

CTDNA

Extracellular nucleic acids in blood plasma were initially documented by Mandel and Metais way back in 1948.[15] Measurement of circulating viral nucleic acids is the standard of care in the

management of many viral infections such as HCV, HIV and CMV. Utilisation of ctDNA may similarly improve the standard of care in cancer patients, as already evident in non-small cell lung cancer patients. ctDNA is fragmented extracellular DNA in blood, derived from tumour cells. Cell-free DNA is the total amount of extracellular DNA in the blood (derived from both normal and tumour cells). This fragmented DNA is thought to be released as a result of apoptosis or necrosis of cells.[16,17]

The cancer cell is a product of serially accumulating genetic alterations and cancer genome sequencing efforts in CRC have identified around a median of 76 non silent mutations that are present in tumour cells but are absent in normal cells[18] DNA harbouring these somatic mutations is highly specific for tumour and can be used to differentiate from other fragmented DNA in blood. The genotyping methods can characterise ctDNA by identifying a tumour-specific mutation(s). And then, mutation can be enumerated in terms of a number of mutant allele copies per mL or as a mutant fraction (%) in an individual sample.

Most commonly used genotyping methods to characterise ctDNA are Polymerase chain reaction (PCR) assay (allele-specific PCR, Emulsion PCR) to look for single or few target mutations/variants and next-generation sequencing (NGS) assay for broader coverage (panel) of mutation hotspots of commonly mutated genes.[19] In addition, epigenetic changes like aberrant DNA methylation specific to tumour genome can also be identified using methods such as methylation-specific PCR.[20] Factors influencing the application of molecular methods for ctDNA analysis are listed in [Table 1]. The concentration of cell-free DNA in serum is higher than plasma. Most of the cell-free DNA in serum results from leukocyte lysis during clotting and this increased contamination may dilute the ctDNA and hamper analytical success.[21] For this reason, plasma is the optimal specimen for analysis of ctDNA, especially if leukocyte fraction is separated soon after the blood draw.[22] As it is a blood-based test, logistic problems of tissue biopsy such as procedural risk and expenses are avoided. The ability to genotype and quantify ctDNA creates a wide array of clinical utility such as screening, diagnosis, prognostication, treatment selection and monitoring of CRC patients during therapy.

CORRELATION OF CTDNA WITH CLINICAL AND BIOCHEMICAL PARAMETERS

Fragmented ctDNA in the blood is released from necrotic or apoptotic cells in tumour. It is reasonable to speculate large volume disease or aggressive histology diseases usually have high cell turnover resulting in high ctDNA. Studies using aberrant DNA methylation to characterise ctDNA have shown, fraction of ctDNA in blood correlate with the stage of disease.[20,23] Similar findings of stage-related correlation were seen when mutation panel was used for ctDNA detection.[24] Within metastatic CRC patients, tumour volume measured using RECIST criteria strongly correlated with the levels of ctDNA.[25,26] Moreover, patients with multiple site metastases had higher ctDNA levels compared to single site metastases.[26] These findings suggest that ctDNA levels mirror the tumour volume and can be used as a surrogate marker for evaluation of tumour burden. Apart from tumour volume, site of metastases also affects ctDNA levels. Patients with liver metastasis had significantly higher ctDNA levels compared to patients without liver metastasis.[26] In contrast, the presence or absence of lung or peritoneal metastasis did not affect ctDNA levels.[26] These findings suggest further evaluation of the other factors that may influence ctDNA levels such as tumour vasculature, cell turnover and ctDNA metabolism.

MINIMAL RESIDUAL DISEASE AND POST-OPERATIVE FOLLOW-UP

Management of localised and resectable metastatic CRC primarily involves surgery with or without chemotherapy

Table 1: Comparison of commonly used ctDNA analysis platforms.[50,51]

ctDNA analysis platforms/Characteristics	Polymerase Chain reaction assays	Next generation sequencing assays
Example	• dd PCR, BEAMing	• Deep sequencing, TAM-seq, Safe-Seqs, CAPP-Seq
Turnaround time	• Fast	• Slow
Cost	• Low cost	• Expensive
Genetic alterations covered	• SNV, indels, CNV • Identify one or few known genetic mutations	• SNV, indels, CNV, rearrangements • Identify novel mutations or multiple mutations in single assay
Quantification/ enumeration	• Absolute quantification of the number of mutant and wild type variants present	• Relative quantification of number of mutant allele copies to wild type allele copies
Sensitivity	• 0.1–0.01%	• 1–0.1%
Interpretation of results	• Easy to interpret	• Complex bioinformatics. Can be limited by false-positive results generated from sequencing artefacts

ctDNA: Circulating tumour DNA, BEAMing: Beads, emulsion, amplification and magnetics, CAPP-Seq: Cancer personalised profiling by deep sequencing, CNV: Copy number variation, ddPCR: Droplet digital PCR, SNV: Single nucleotide variation, TAM-seq – Tagged-amplicon deep sequencing

(± radiotherapy in rectal cancers).[6] Benefits of adjuvant therapies in Stage II colon cancer are not conclusively demonstrated and currently indicated in high risk Stage II patients. Despite the curative surgery and perioperative therapy around one in three Stage II and Stage III, patients eventually have disease recurrences.[27] Early detection of recurrence results in increased chances of curative resection and improved overall survival.[5] At present, standard of care is to follow up with computed tomography (CT) imaging and tumour marker Carcinoembryonic antigen (CEA). There is growing interest to optimise surveillance strategy and search for sensitive novel biomarker for evaluating occult residual or recurrent disease.

Molecular technologies have improved the ability to pick up residual disease not detectable by conventional methods in various haematological malignancies. Detection of ctDNA following surgery may suggest the presence of occult residual disease, as half-life of ctDNA in blood ranges from 15 min to few hours.[13,16] Ryan *et al*. reported that in a significant proportion of operable CRC patients, ctDNA can be detected preoperatively and persistent elevation postoperatively strongly correlated with the disease recurrence.[28] In another study conducted exclusively in Stage II, CRC patients (without adjuvant chemotherapy) suggested that detectable ctDNA postoperatively was associated with markedly increased risk of recurrence compared to patients with no detectable postoperative ctDNA.[29] Moreover, in those who received adjuvant chemotherapy, the presence of ctDNA after completion of chemotherapy was also associated with a significantly shorter recurrent-free survival.[29] A recently published prospective biomarker study involving 159 locally advanced rectal cancer patients were monitored serially with ctDNA. Among 159 patients, 77% of pre-treatment and 12% post-surgery plasma samples were positive for ctDNA. The estimated 3-year recurrence-free survival varied from 87% for the post-operative ctDNA-negative patients to 33% for the post-operative ctDNA-positive patients.[30]

Even in the subset of resectable metastatic CRC patients, persistence of post-operative ctDNA levels was associated shorter recurrence-free survival.[24,31,32] And also in resectable metastatic CRC complete resection was associated with 99% median fall in immediate post-operative ctDNA.[32] These studies also showed that serial ctDNA analysis could pick up recurrent disease months before CT imaging and CEA and more sensitive indicator than CEA.[31,32] These findings suggest that ctDNA may add to conventional methods in the post-operative follow-up, to detect residual or recurrent disease. Studies assessing the utility of post-operative ctDNA analysis to recurrence are listed in [Table 2].

MOLECULAR CHARACTERISATION TO AID IN TREATMENT SELECTION

Tumour molecular profiling for KRAS, NRAS and BRAF mutation status is standard of care in metastatic CRC patients for prognostication and selection of therapy.[6] Mutations in the RAS pathway predict a lack of response to anti-EGFR therapy. Studies evaluated whether ctDNA molecular profiling reliably reflects the tumour tissue molecular profiling in CRC patients. These studies have varied rates of concordance and prognostic significance between tumour and blood.[24,33-35] Inconsistencies in testing methodologies, the time intervals, clinical settings and intratumoral heterogenicity may be the probable reasons for differing concordance rates between studies. In Kidess *et al.* study, concordance between tissue and plasma for KRAS and BRAF mutation improved from 53% for a non-metastatic disease to 93% for metastatic disease.[24] In addition, in three metastatic patients, PIK3CA mutation was detected only in plasma.[24] Xu *et al*. evaluated in chemotherapy naïve patients showed a 73% concordance rate and also revealed that KRAS mutation was associated with poor prognosis irrespective of the source of mutational detection.[34] Thierry *et al*. evaluated 95 metastatic CRC patients and reported 96% concordance between plasma and tumour tissue for KRAS and BRAF.[35] [Table 3] shows the validation studies of the RAS pathway mutations in plasma compared to tumour tissue.

Siravegna *et al*. evaluated 100 metastatic CRC patients to assess tissue and plasma concordance of RAS pathway mutations (KRAS, NRAS and BRAF) using Droplet Digital PCR for ctDNA analysis. Out of 100 cases, 61 patients had RAS pathway mutations and 36 did not have mutations resulting 97% concordance rate.[36] In the PROSPECT-C prospective Phase II study, significant proportions of RAS wild type metastatic CRC patients on tissue evaluation had RAS pathway mutations in pre-treatment ctDNA and these patients did not benefit from anti-EGFR therapy.[37] These studies suggest that molecular profiling ctDNA can be a reliable substitute for a tissue, at least in the setting of insufficient sample and also may provide additional information.

Another crucial issue in the molecular profiling of ctDNA is the role of target mutation panel in the management of CRC. Siravegna *et al*. evaluated plasma DNA of eight CRC patient's upfront refractory to anti-EGFR therapy. All these eight patients had wild type RAS pathway genes in both plasma and tissue. CtDNA analysis with 226 gene NGS panel revealed four (50%) of eight patients had ERBB2 amplification.[36] Another study analysed plasma ctDNA of metastatic CRC patients treated with dual anti Her2neu therapy in the Phase II HERACLES trial. Compared to responders, majority among non-responders had RAS pathway mutation at baseline. Many responders developed mutations involving

Table 2: Key studies evaluating the role of post-operative ctDNA analysis to predict recurrence risk.

Author, Year of publication	Type of study/ Sample size (*n*)	Target/ctDNA analysis method	Stage	Post op ctDNA positive rates (%)	Recurrence rate among positive post op ctDNA group	Hazard ratio for recurrence
Ryan *et al.* 2003[28]	Prospective/94	KRAS/Direct sequencing	TVA, Dukes A–D	17% (16/94)	63% (10/16)	6.37 (95% CI 2.26–18.0; *P*=0.001)
Diehl *et al.* 2008[32]	Prospective/18	APC, KRAS, PIKC3A, TP53/ BEAMing	IV¥	80% (16/20)	93.75% (15/16)	All except one recurred in positive compared to none in the negative (P=0.006)
Tie *et al.* 2016[29]	Prospective/230	Somatic mutation with the highest MAF/PCR with Safe-seqS	II (without adjuvant chemotherapy)	7.9% (14/178)	78.6% (11/14)	18 (7.9–40, *P*<0.001)
			II (with adjuvant chemotherapy)	6.8% (3/44)	66.6% (2/3)	11 (1.8–68, *P*=0.001)
Schøler *et al.* 2017[31]	Prospective/44	Panel of SSVs and SPMs/ddPCR	I–III	28.5% (6/21)	100%	37.7 (4.2–335.5, *P*<0.001)
			IV¥	30.4% (7/23)	100%	4.9 (1.5–15.7, *P*=0.007)
Tie *et al.* 2019[30]	Prospective/159	Somatic mutations in 15 recurrently mutated genes/ PCR with Safe seqS	II–III#	12% (19/159)	58% (11/19)	HR 13 (95% CI 5.5–31, *P*<0.001)

¥: Resectable metastatic CRC, #: Stages II and III carcinoma rectum. BEAMing: Beads, emulsion, amplification and magnetics, ddPCR: Droplet digital polymerase chain reaction, MAF: Mutant allele frequency, SPMs: Single point mutations, SSVs: Single structural variants, TVA: Tubulovillous adenoma, ctDNA: Circulating tumour DNA

Table 3: Validation studies of the RAS pathway mutations in plasma compared to tumour tissue.

Author, Year of publication	Type of Study	Number of patients	ctDNA analysis Method	Target	Results
Kidess *et al.*, 2014[24]	Retrospective	38	Multiplexed SCODA mutation enrichment and detection assay	KRAS BRAF EGFR PIK3CA	Concordance rates 53% and 93% for non-metastatic and metastatic disease respectively
Xu *et al.* 2014[34]	Retrospective	242	PNA PCR	KRAS	Concordance rate 73% (K=0.456)
Thierry *et al.*, 2014[35]	Blinded prospective	95	AS qPCR	KRAS BRAF	Concordance rates 96%
Siravegna *et al.* 2015[36]	Prospective	100	dd PCR, BEAMing	KRAS NRAS BRAF	Concordance=97%

AS Qpcr: Allele specific quantitative polymerase chain reaction, ddPCR: Droplet digital polymerase chain reaction, PNA PCR: Peptide-nucleic-acid-mediated polymerase chain reaction, SCODA: Sequence-specific synchronous coefficient of drag alteration assay, ctDNA: Circulating tumour DNA

the PIK3-AKT pathway at progression.[38] In another study, analysis of plasma ctDNA of 78 metastatic CRC patients using targeted NGS panel showed 69.2% of patients harboured at least one actionable alteration. Moreover, specific genetic alterations such as amplifications in *BRAF, KRAS, PIK3CA, MET* and *FGFR2* were only detected in ctDNA.[39] These results suggest that the ctDNA mutation panel done serially may provide relevant additional information to single tissue-based molecular profiling. Prospective randomised trials are required to translate these findings for routine clinical use in the treatment modifications of metastatic CRC.

MONITORING RESPONSE TO THERAPY IN ADVANCED CRC.

High percentage of patients (about 90%) have detectable ctDNA at baseline in metastatic CRC patients.[25,26] The ability to genotype and enumerate tumour mutations makes ctDNA assay the optimal tool for real-time monitoring of tumour dynamics. In a small prospective study, fall in ctDNA after one cycle of chemotherapy correlated with CT responses after four cycles and patients having >10-fold reduction had longer PFS.[25] In another study, more than 50% reduction

Table 4: Advantages and limitations of ctDNA in CRC.

Advantages	Limitations
Sample acquisition: Easy, quick, minimal procedural risk to the patient, less expensive	Requires special processing and handling unless using cell-stabilisation tubes
Comprehensive tissue profile (reflection of inter- and intra-tumour heterogeneity)	Low correlation with histology or cellular phenotype
Correlates with tumour burden	Limited data on confounding patient-related factors
Can be done serially and provides real time information (Time-course profile)	Limited evidence to change therapy at the time of ctDNA progression
Can be used to detect minimal residual disease and risk of recurrence	No prospective randomised trial to test the impact on survival outcomes
Can complement conventional methods for monitoring therapy (Monitoring Tumour Dynamics with therapy)	No cost benefit analysis compared to standard of care
Can detect resistant clones early and may predict progression	High-sensitivity ctDNA assays may detect subclonal variants or passenger mutations that may misguide therapy
	Limited evidence for treatment selection

CRC: Colorectal cancer, ctDNA: Circulating tumour DNA

during second-line chemotherapy associated with longer median PFS and OS.[40] Similarly, ctDNA levels mirrored image response criteria in metastatic CRC patients on dual anti-Her2neu therapy.[38] Image-based RECIST response criteria have certain limitations such as inapplicability to non-measurable disease and inability to differentiate between live neoplastic cells, dead neoplastic cells and varying amounts of non-neoplastic cells. Although studies included small sample size, early detection, ability to pick up the occult disease, no ionising radiation and better reproducibility offer an advantage over conventional methods.

Serial liquid biopsies may also provide crucial information on the development of resistant clones under therapeutic pressure and predict progression early. For example, serial ctDNA assessments in a patient receiving anti EGFR-targeted therapy showed evolving resistant mutations involving EGFR and KRAS or MET gene amplification. These resistant clones can be detected months before radiological progression.[36,41] Moreover, these resistant clones may decline on withdrawal of anti-EGFR therapy, giving the second opportunity to re-challenge.[42] It will be interesting to see whether stopping and restarting anti-EGFR therapy based on ctDNA analysis impact survival outcomes.

PROGNOSTICATION

Cell-free DNA levels (allele copies/ml of plasma) are higher in CRC patients than in normal individuals or individuals with other comorbidities.[43] Quantification studies evaluating the relevance of higher baseline cell-free DNA have shown poor survival outcomes in metastatic CRC patients.[43,44] However, contamination due to lysis of leukocytes may vary cfDNA levels and limits the utility of these assays for prognostication. Detection of KRAS mutations in plasma at baseline are also associated with worse survival outcomes.[44,45] Similarly, in a prospective randomised Phase III CORRECT trial, the exploratory analysis revealed shorter median PFS and OS for patients with higher baseline cfDNA and KRAS mutations in plasma. Targeted NGS ctDNA panel for frequently mutated genes in CRC improves sensitivity for the detection of ctDNA. Metastatic CRC with liver metastases and high disease burden is associated with higher ctDNA levels burden.[25,26] This suggests quantification of ctDNA at baseline may provide prognostic information as it reflects disease burden.

SCREENING

Detection of cancer in the pre-symptomatic phase is an ongoing need in any cancer, including CRC, as it may result in less intensive treatment and higher cure rates. Colonoscopy and faecal occult blood tests are the current screening methods for colon cancer, either invasive or insensitive, in an early stage. Ultrasensitive ctDNA assay theoretically offers the advantage of finding the disease at an early stage. However, detection of low-frequency somatic mutation or variant through screening in the general population may be confounded by clonal haematopoiesis of indeterminate potential (CHIP).[46] Finding highly prevalent CHIP will result in higher false-positive rates and morbidity of further workup.

Aberrant methylation of CpG islands in the promoter region of tumour suppressor genes occurs early in tumorigenesis, more prevalent and the profiles of methylation are specific to the tumour types.[47] Church *et al.* prospectively evaluated 7941 individuals aged ≥50 years old undergoing screening colonoscopy with methylated SEPT9 DNA and found a crude sensitivity rate of 50.9% for all CRC stages and sensitivity was as low as 35% for Stage I CRC.[48] Luo *et al.* evaluated 1493 high-risk patients with colonoscopy and cg10673833

methylation testing. The cg10673833 methylation testing identified 26 of 29 CRC patients, with a sensitivity of around 90% and specificity of 86.8%.[49] However, around 12% of participants without CRC also tested positive. These results are promising but require further randomised studies before translating into clinical practice.

Limitations

As ctDNA analysis is newer and emerging, the data are limited. Liquid biopsy provides very little information on the histological phenotype. Although liquid biopsy appears to cater to precision medicine better, there is a need for prospective randomised clinical trials before translating to routine practice, as most of the studies are retrospective analyses involving a small sample size. The advantages and limitations are summarised in [Table 4].

Future perspectives

As has happened in lung cancer, the management has evolved and significant contributions were made due to understanding of molecular nature of disease, the horizon has opened up in colon cancer too. Certainly, prospective studies are limited in colon cancer. There is a need for large scale studies to address the role of ctDNA in colon cancer.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Conflicts of interest

There are no conflicts of interest.

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