Evolving Applications of Liquid Biopsies in Gastrointestinal Cancers

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Abstract: Liquid biopsy is a test that allows for the diagnosis and analysis of cancer by sampling cancer cells or byproducts present in biological fluids such as blood or urine. It has the potential to create a new paradigm in oncologic care, being a less invasive approach than conventional tissue biopsy. Liquid biopsy has multifaceted applications for longitudinal disease monitoring in terms of surveillance, treatment response, and identification of emerging resistance mechanisms. Multiple assays currently exist or are in development for detecting circulating tumor cells, DNA, RNA, exosomes, proteins, fragmentomic markers, and metabolomes. Here, we review the applications of liquid biopsy in gastrointestinal cancers, emphasizing its use in both perioperative and advanced settings. We also examine its role in screening, diagnostics, and other cancer-related scenarios.

Keywords
ctDNA, gastrointestinal malignancies, liquid biopsy

Introduction

Despite diagnostic and therapeutic advances, gastrointestinal (GI) cancers are the leading cause of cancer-related mortality, a trend anticipated to persist for decades to come.[1] Many patients experience relapse after curative procedures, and the 5-year survival rates for advanced-stage disease range from 14.6% for colorectal cancer (CRC) to 3.2% for pancreatic ductal adenocarcinoma (PDAC).[2] Tumor molecular profiling is crucial to GI cancer management, particularly in patients with advanced-stage disease. The results of profiling inform the use of US Food and Drug Administration (FDA)-approved targeted therapies, which can improve patient survival, as well as help identify patients for clinical trials.[3] Tumor

immune microenvironment profiling and multiomics analyses, including tumor genomics, transcriptomics, epigenetic modifications, and protein signature analyses, have further contributed to characterizing tumors and prognosticating outcomes.[4]

Liquid biopsies have emerged as a highly attractive tool in both research and clinical practice. Circulating tumor DNA (ctDNA), intact circulating tumor cells (CTCs), secreted tumor proteins, and extracellular vesicles are various tumor components that are detectable in blood.[5] This allows for genomic profiling, capturing temporospatial molecular heterogeneity, monitoring tumor dynamics, studying treatment resistance mechanisms, and detecting microscopic disease—all now feasible through the sampling of peripheral blood.

There has been an exponential rise in the use and study of liquid biopsies in GI cancers. Most studies to date have assessed the ability of ctDNA to characterize tumor genomics and detect measurable residual disease (MRD). Data are emerging on its ability to assess treatment responses and diagnose cancers that are not amenable to biopsy or of unknown primary origin.[1,6-9] CTCs have been studied as prognostic and predictive biomarkers, and ex vivo propagation can facilitate the development of xenograft or organoid models.[10,11] Multiomics and extracellular vesicle studies, although in their infancy, are also potential promising biomarkers.[12] Although there is tremendous potential for the use of liquid biopsy, much remains unknown. Testing is nonstandardized, and the limitations of these technologies must be considered. Herein, we review the current understanding and future directions of liquid biopsy clinical applications in patients with GI cancers.

Circulating Tumor DNA

Cell-free DNA (cfDNA) refers to cell-derived DNA fragments that are present in the blood via active processes (eg, release of extracellular vesicles) and passive processes (eg, apoptosis).[13] ctDNA is cancer-derived cfDNA harboring tumor-specific mutations and epigenetic modifications. Levels can range from less than 0.1% to more than 90% of cfDNA, depending on stage, anatomic location, and histology.[13] ctDNA is cleared via excretion or reuptake by organs (eg, liver, spleen, kidneys), phagocytic clearance, or enzymatic degradation by deoxyribonuclease, all of which render its half-life to less than 2 hours.[14] This short half-life makes ctDNA a tool for dynamic, real-time tumor characterization.

ctDNA can be detected, quantified, and characterized through various assays ranging from hot-spot single-gene or multi-gene polymerase chain reaction (PCR)-based assays or broad next-generation sequencing (NGS), all of which exhibit relatively high sensitivity and specificity. Because the amount of DNA captured in peripheral blood is lower than that from tissue biopsies, various methodologies have been developed to sequence low levels of DNA, such as digital droplet PCR (ddPCR); beads, emulsion, amplification, and magnetics (BEAMing); tagged-amplicon deep sequencing (TAm-Seq); or cancer personalized profiling by deep sequencing (CAPP-Seq). Each methodology has its limits in ctDNA detection, sensitivity, and specificity, but undergoes constant optimization to improve its depths of detection.[15]

ctDNA assays can be tumor-informed or tumor-agnostic. Tumor-informed assays detect the presence of known genetic mutations from a patient's tumor. By leveraging a greater depth of sequencing for a small number of tumor-specific genes, they have higher sensitivity in detecting very low levels of DNA, making them particularly attractive in detecting MRD.[16] However, manufacturing time for these personalized assays can take 1 to 2 months. Tumor-agnostic assays sequence the plasma for common cancer-related gene alterations and do not require a tumor biopsy. They also have a shorter turnaround time of 7 to 10 days. Although tumor-agnostic assays can comprehensively characterize various alterations across many genes, the depth and sensitivity of sequencing at each gene may be compromised.[15] Therefore, this approach is often favored for characterizing more advanced disease with higher baseline ctDNA levels. Both tumor-agnostic and tumor-informed assays can be used in the MRD setting, but no studies have compared them directly against each other.

ctDNA in the Perioperative Setting

Numerous studies using tumor-informed assays at various times after surgery have demonstrated ctDNA sensitivities of 48% to 100%, specificities of greater than 90%, and positive predictive values nearing 100% for detecting relapse, with median lead times of detecting radiographic recurrence of 4 to 10 months.[8,17,18] Tumor-uninformed assays have also shown promise in the postoperative setting.[19] Additionally, other studies have demonstrated that postsurgical ctDNA positivity rates may mirror the expected relapse rates by clinical stage, highlighting the ability of ctDNA to predict relapse.[20,21]

In CRC, a prospective study by Henriksen and colleagues using a tumor-informed assay in 160 patients with stage 3 CRC showed that patients with positive ctDNA 2 to 4 weeks after surgery (hazard ratio [HR], 7.04; $P<.001$), after adjuvant treatment (HR, 50.76; $P<.001$), and on longitudinal testing (HR, 50.80; $P<.001$) had a much higher risk of relapse than patients with ctDNA-negative results at those time points.[22] Parikh and colleagues used a tumor-uninformed assay to show that ctDNA positivity 1 month after surgery was prognostic for relapse in stages

1 to 4 CRC (HR, 11.28; *P*<.0001), with a specificity of 100% and sensitivity of greater than 90% with serial testing.[19]

Tie and colleagues conducted a prospective trial of 455 patients with stage 2 CRC, a group in which most patients are cured with surgery alone and are potentially overtreated with chemotherapy. Patients were randomized in a 2:1 ratio to a ctDNA-guided adjuvant management arm (if a postoperative 4- or 7-week ctDNA was positive, patients in this arm would receive oxaliplatin- or fluoropyrimidine-based chemotherapy; if negative, no treatment) or to a standard-of-care (SOC) arm.[23] Fewer patients in the ctDNA-guided arm received adjuvant therapy (15% vs 28%), and the primary endpoint of 2-year recurrence-free survival (RFS) was noninferior to the SOC arm (93.5% vs 92.4%). Exploratory analyses showed that ctDNA-negative, low-risk (T3) patients had better 3-year RFS than ctDNA-negative, high-risk (T4) patients (96.7% vs 85.1%; HR, 3.04; 95% CI, 1.01-6.71). These findings support using a ctDNA-guided approach in stage 2 patients to spare some patients of adjuvant chemotherapy and its toxicities without compromising outcomes.

In the prospective, observational Japanese GALAXY study, patients with stages 2 or 3 CRC who were tumor-informed ctDNA-positive 4 weeks after surgery had worse 12-month RFS than those who were negative by ctDNA (55.5% vs 95.2%; HR, 13.3; 95% CI, 8.0-22.2; *P*<.001).[24] Among ctDNA-negative patients, there was no significant difference in 6- or 12-month RFS with or without adjuvant therapy. Among 838 patients with stages 1 to 4 disease, those with ctDNA remaining negative between 4 and 12 weeks after surgery and those with ctDNA converting from positive to negative had excellent 6-month RFS rates (98.0% and 100%, respectively) vs those with ctDNA converting from negative to positive or persistently positive (62.5% and 58.3%, respectively). Although limited by short follow-up and nonrandomized study design, these early findings suggest that ctDNA-negative patients may not derive additional benefit from adjuvant therapy, and clearance of ctDNA in high-risk positive patients may improve survival outcomes.

Post hoc analysis of the IDEA trial, in which patients with stage 3 CRC were randomized to 3 vs 6 months of adjuvant therapy, showed that ctDNA was prognostic for disease-free survival in patients treated for 3 months but not in those treated for 6 months, highlighting its potential to risk stratify patients for longer durations of adjuvant therapy.[25] The optimal time after surgery to check ctDNA is unclear, but many providers test between 4 and 6 weeks after surgery, and some data suggest that it should be done as early as 2 weeks after surgery.[18]

Another clinical dilemma is whether to offer adjuvant chemotherapy after metastasectomy. ctDNA is prognostic after metastasectomy, and ongoing studies are examining the utility of ctDNA in identifying patients who are most likely to benefit from adjuvant therapy.[26,27] In rectal cancer, where nonoperative approaches are increasingly considered, there are conflicting results regarding the ability of ctDNA to predict pathological complete responses (ypCR) after chemoradiation.[28] Further validation is required before using it to guide decisions for pursuing watch-and-wait vs surgery. Randomized, prospective trials are needed to validate ctDNA as a predictive biomarker before it can be routinely used to guide therapy escalation or deescalation (Table 1).

In upper GI cancers, several observational studies have demonstrated that the presence and quantity of baseline, postneoadjuvant, and postoperative ctDNA were associated with risks of recurrence and survival.[29-34] In a biomarker analysis of 50 patients enrolled in a perioperative gastric cancer trial, patients with major responses after neoadjuvant therapy had higher preoperative ctDNA-negative rates (100%) vs those without major responses (56%).[35] In another study, similar trends were seen with ypCR in esophageal squamous cell carcinoma patients based on preneoadjuvant ctDNA results.[36] Although it is not validated to predict ypCR today, when combined with other diagnostics, it has the potential to determine the need for completion surgery.

In PDAC, multiple studies have demonstrated the prognostic value of ctDNA both pre- and postoperatively, often using assays to detect plasma *KRAS* mutations.[37] Botta and colleagues reported that at both times, ctDNA positivity correlated with clinical stage and RFS, outperforming CA 19-9 as a prognostic marker.[38] Promising results were reported from a phase 1 study of a KRAS peptide vaccine in patients with *KRAS* G12D/G12R–mutated PDAC or CRC with MRD as detected by tumor markers CA19-9/CEA or ctDNA *KRAS/NRAS* mutations.[39] The treatment was safe, and biomarker reduction and MRD clearance were achieved in 79% and 21% of patients, respectively. Other studies have shown that ctDNA is prognostic in hepatobiliary cancers.[40] Although longer follow-up with survival data is needed, studies in anal squamous cell carcinoma showed that patients who achieved ctDNA clearance also had a clinical complete response after definitive chemoradiation.[41] Although some data in GI stromal tumors correlate ctDNA with tumor size and disease activity, robust observational studies in the MRD setting are lacking.[42]

Although ctDNA is a validated prognostic biomarker, prospective randomized trial results are needed to validate ctDNA as a predictive biomarker to guide perioperative treatment decisions (Tables 1 and 2). If ctDNA can ultimately be validated as a surrogate marker for survival, it has the potential for use as an early readout

Table 1. Prospective ctDNA-Based Therapeutic and Surveillance Intervention Trials in the MRD Setting for CRC

Trial, phase	Patient population	Design	Intervention	Primary endpoints	Secondary endpoints
NCT04089631 (CIRCULATE), 3	Stage II CRC, following resection	Randomized	Randomized to the following based on postoperative ctDNA result: If ctDNA+: Arm 1: AC with cape ± oxaliplatin; Arm 2: no chemotherapy, follow-up within trial If ctDNA–: Arm 3: follow-up within study; Arm 4: standard follow-up outside study	DFS in ctDNA+ group	OS in ctDNA+ patients with or without AC DFS and OS in ctDNA+ patients vs ctDNA–
NCT04068103 (COBRA), 2/3	Stage IIA CRC, following resection	Randomized	Randomized to: Arm 1: active surveillance Arm 2: prospective ctDNA-based treatment: if ctDNA+: AC with FOLFOX for 6 months; if ctDNA–: active surveillance	ctDNA clearance for ctDNA+ patients (phase 2), RFS in ctDNA+ patients (phase 3)	RFS, OS, TTR, adherence, incidence rate of ctDNA positivity after resection, quantitative ctDNA, genomic profile, cost effectiveness of ctDNA vs SOC
NCT04120701 (CIRCULATE PRODIGE), 3	Stage II CRC, ctDNA+ following resection	Randomized	Randomized to: mFOLFOX6 No intervention	3-y DFS	2-y DFS, OS, AEs
NCT05031975 (ERASE-TMZ), 2	Stage II/III MGMT methylated CRC, ctDNA+ after resection and AC	Single-arm	6 cycles of TEMIRI	Seroconversion after TEMIRI consolidation	3-y DFS, 3-y OS, safety, QOL
NCT04486378, 2	Stage II/III CRC, ctDNA+ following resection	Randomized	Randomized to: RO7198457 (autogene cevumeran personalized vaccine) Observation	5-y DFS	RFS, TTR, TTF, OS, change in ctDNA every 3 mo, TEAEs, dose adjustments
NCT04050345 (TRACC)	Stage II/III CRC, ctDNA+ following resection	Observational/randomized	Randomized to: SOC arm ctDNA informed arm If ctDNA-: de-escalation of AC (chemotherapy and duration) If ctDNA+: SOC	3-y DFS ctDNA guided chemo vs SOC	ctDNA detection before, during, and after treatment
NCT05529615	Arm 1: high-risk, stage II/low-risk or stage III CRC Arm 2: high-risk, stage III CRC following resection	Randomized	Arm 1 (high-risk stage II, low-risk stage III): ctDNA measured at 7 days after surgery If ctDNA–: observation If ctDNA+, 1:1 randomization: 3 mo CAPOX vs observation Arm 2 (High-risk stage III): ctDNA after CAPOX for 3 mo If 3-mo ctDNA–: observation If 3-mo ctDNA+: 1:2 randomization to 3 more mo of CAPOX vs second-line	3-y DFS	

Table 1. (*Continued*) Prospective ctDNA-Based Therapeutic and Surveillance Intervention Trials in the MRD Setting for CRC

Trial, phase	Patient population	Design	Intervention	Primary endpoints	Secondary endpoints
NCT05534087 (CLAUDIA), 3	Stage II/ III CRC, ctDNA+ following resection	Randomized	Randomized to: 6 cycles of mFOLFIRINOX, 6 cycles of FOLFOX, or 4 cycles of CAPOX	3-y DFS	ctDNA clearance rate, 5-y OS, TRAEs, QOL
NCT05350501 (CLAUDE), 2	Stage II/ III CRC, ctDNA+ following curative therapy	Single-arm	EO2040 (microbiome-derived therapeutic vaccine) and nivolumab	ctDNA clearance at 6 mo	Safety, response at 3 mo, DFS, OS
NCT05062889 (ERASE-CRC), 2	Stage II/ III CRC, ctDNA+ following resection	Randomized	Randomized to: FOLFOX for 12 cycles/CAPOX for 8 cycles FOLFOXIRI for 12 cycles If ctDNA+ after AC, randomized to: Observation Post-adjuvant trifluridine/ tipiracil for 6 cycles	ctDNA clearance after adjuvant treatment, ctDNA clearance after post-adjuvant treatment	Toxicity, DFS, OS
NCT04084249 (IMPROVE-IT2)	Stage II/ III CRC following resection	Nonrandomized	Arm 1: ctDNA-guided surveillance. Perform ctDNA analysis every 4 mo. At time of first ctDNA+ test, patients undergo PET/CT and colonoscopy. If negative for recurrence, repeat surveillance PET/CT every 3 mo for 21 mo or until recurrence detection. Arm 2: standard surveillance. CT scan at 12 and 36 mo and colonoscopy every 5 y. ctDNA blood samples measured retrospectively	Fraction of patients with relapse receiving intended curative resection or local treatment for complete eradication	3-y and 5-y OS, time to clinical recurrence, time to molecular recurrence, QOL, fear of cancer recurrence inventory, impact of Events Scale - Cancer (15-question survey regarding cancer-specific distress), cost-effectiveness
NCT05174169 (CIRCULATE-US), 2/3	Stage III CRC following resection	Randomized	Randomized to the following based on postoperative ctDNA result: If ctDNA+: mFOLFOX6 for 6 mo mFOLFIRINOX If ctDNA–: mFOLFOX6 3-6 mo Observation with serial ctDNA monitoring	ctDNA+ status, DFS	Baseline post-surgery ctDNA positivity rate, OS, recurrence, adjuvant chemotherapy adherence
NCT04457297 (ALTAIR), 3	Stage III CRC, ctDNA+ following resection and AC	Randomized	Randomized to: Placebo Arm 2: trifluridine/tipiracil	DFS	Rate of ctDNA clearance, OS, AEs, QOL
NCT05343013, 2	Stage II-IV CRC, ctDNA+ following resection and AC	Single-arm	TAS-102 for maintenance	6-mo ctDNA clearance rate	3-mo ctDNA clearance rate, DFS, OS, safety, response markers, ctDNA profile changes, predictive biomarkers

Table 1. (*Continued*) Prospective ctDNA-Based Therapeutic and Surveillance Intervention Trials in the MRD Setting for CRC

Trial, phase	Patient population	Design	Intervention	Primary endpoints	Secondary endpoints
NCT04920032, 1b	Stage II-IV CRC, ctDNA+ after all SOC therapy	Single-arm	TAS-102 + irinotecan	Incidence of ctDNA+ after 6 mo treatment	Grade 3-5 AEs
NCT05900648 (RX-CROME), 2	Stage II-IV CRC, ctDNA+ after all SOC therapy	Single-arm	6 mo of regorafenib and vudalimab (XmAb20717)	ctDNA clearance at 6 mo	3-mo ctDNA clearance, DFS, OS, safety/tolerability
NCT05062317 (REACT-CLM), 2	Stage IV CRC with liver metastases after hepatectomy	Nonrandomized	If ctDNA–: capecitabine or 5-FU If ctDNA+: FOLFOX/FOLFIRI +/- bevacizumab	1-y RFS in ctDNA– patients	1-year RFS in ctDNA+, OS in ctDNA+ and ctDNA– groups, ctDNA– rate at 1 y post resection, chemo change based on ctDNA dynamics, pattern of recurrence, ctDNA, sensitivity and specificity for recurrence, PRO, correlative characterization upon ctDNA detection, AE
NCT03803553, 3	Stage III CRC following resection	Parallel assignment	If ctDNA+, randomized to: FOLFIRI Active surveillance with serial ctDNA If ctDNA–: active surveillance If ctDNA+ MSI-H: nivolumab If ctDNA+ *BRAF*-mut: encorafenib/binimetinib/cetuximab	DFS ctDNA clearance	OS, ctDNA clearance in nivolumab arm, DFS in nivolumab arm, ctDNA clearance in BRAF arm, DFS in BRAF arm, ctDNA as surrogate marker, time to recurrence
NCT05036109 (DAILY)	ctDNA+ CRC with NED	Single arm	Aspirin + vitamin D + behavioral/diet/exercise support	ctDNA clearance rate after 3 mo of lifestyle interventions	Dynamics of ctDNA after 3 mo, 1-y recurrence rate
NCT05040568, 1b	MRD+ resected CRC	Single arm	CB-NK cells + cetuximab	Activity of CB-NK with MRD+	

5-FU, 5-fluorouracil; AEs, adverse events; AC, adjuvant chemotherapy; CAPOX, capecitabine and oxaliplatin; CB, cord blood; ctDNA, circulating tumor DNA; CRC, colorectal carcinoma; CT, computed tomography; DFS, disease-free survival; EORTC, European Organisation for Research and Treatment of Cancer; FOLFIRI, leucovorin, 5-fluorouracil, and irinotecan; FOLFOX, leucovorin, 5-fluorouracil, and oxaliplatin; MGMT, O6-methylguanine-DNA methyl-transferase; mo, months; MRD, measurable residual disease; MSI-H, microsatellite instability–high; mut, mutated; NED, no evidence of disease; NK, natural killer; OS, overall survival; PET, positron emission tomography; PFS, progression-free survival; PRO, patient-reported outcome; RFS, recurrence-free survival; SOC, standard of care; QOL, quality of life; TEMIRI, temozolomide and irinotecan; TRAEs, treatment-related adverse events; TTF, time to treatment failure; TTR, time to recurrence; y, year.

of therapeutic efficacy that may one day facilitate timely drug development and regulatory approvals.

ctDNA as a Biomarker in Advanced Disease

ctDNA can be used to assess treatment responses and/or serve as a harbinger for emerging resistance in advanced disease. Parikh and colleagues demonstrated that metastatic GI cancer patients who had a decline in ctDNA of at least 30% using a tumor-informed assay at 4 and 8 weeks after initiation of systemic therapy had a longer progression-free survival (PFS) than patients who did not meet this threshold, and ctDNA outperformed traditional

tumor biomarkers in predicting treatment response.[43] In the PLACOL study, in which ctDNA was drawn before cycles 1 to 3 of chemotherapy in the first- or second-line setting, patients with higher baseline ctDNA concentrations (>10 ng/mL) had a shorter overall survival (OS) than those with low concentrations (≤10 ng/mL), at 6.8 vs 33.4 months, respectively.[44] Additionally, those with a decrease in ctDNA of at least 80% before cycles 2 or 3 had better overall response rates, PFS, and OS than those who did not. Similarly, in gastroesophageal cancer, ctDNA dynamics have been studied to monitor response and potentially identify poor prognostic genomic alterations.[29,30] For hepatocellular carcinoma (HCC), liquid biopsies are being explored as a means of noninvasive testing and to identify important prognostic mutations and their variant allele frequencies (VAFs) that can be associated with treatment resistance and decreased survival.[40] For biliary tract cancers, higher baseline ctDNA VAFs were associated with worse survival and chemotherapy response.[6,45] In PDAC, studies have shown that the number of plasma alterations detected are prognostic for PFS and OS, that *KRAS* and *TP53* ctDNA kinetics are predictive of treatment response, and that ctDNA outperforms CA19-9 as a predictor of early treatment response and tumor progression.[46-48] Numerous other studies have shown the prognostic value of ctDNA detection, concentrations, and kinetics in non-CRC malignancies.[6,29-34,40,45,46,48,49]

In a landmark phase 2 study, Bratman and colleagues used a tumor-informed assay to evaluate the response of 94 patients with mixed solid tumors to pembrolizumab. ctDNA downtrends between blood drawn pretreatment and before cycle 3 of pembrolizumab were associated with improved PFS and OS.[9] Complete or partial radiographic responses were associated with a ctDNA decrease or clearance, whereas radiographic progression was associated with increasing ctDNA levels. This study supports ctDNA as a predictor of immunotherapy response, and further study might prove ctDNA useful in distinguishing between radiographic pseudoprogression and true progression. These observational studies show the potential role of ctDNA in guiding therapy and sparing patients unnecessary treatment toxicities.

Molecular Characterization in Advanced Disease

Numerous studies have evaluated the performance of ctDNA assays to characterize the tumor molecular landscape and identify actionable mutations. The concordance rates between plasma and tissue biopsies of GI cancers can range from 20% to 100%.[6,7,45,46,50-53] Concordance is largely dependent on tumor shedding, which varies based on tumor type, tumor burden, VAFs, intratumor heterogeneity, and site(s) of metastases, and tends to be higher in more advanced disease.[7,54,55] ctDNA can detect mutations not otherwise identified in primary tumors, highlighting its ability to capture intratumor and intertumor heterogeneity.

ctDNA as a predictive biomarker for GI cancer response and emerging resistance to treatment can be exemplified by the use of anti-EGFR therapy in *RAS*–wild type metastatic CRC (mCRC).[56,57] Topham and colleagues found 29 acquired genetic mutations in ctDNA of mCRC patients following anti-EGFR therapy.[58] However, resistant clones can exponentially decay over time after cessation of therapy, thereby allowing for a clinically meaningful rechallenge, as demonstrated in the CRICKET and CHRONOS studies.[57,59,60] The incidence of "Neo*RAS*" mCRC, a phenomenon in which *RAS*-mutated mCRC is converted into *RAS*–wild type mCRC through eradication of *RAS*-mutated clones, was evaluated using a tumor-agnostic assay and reported at 9.8%, highlighting another potential group that could benefit from anti-EGFR therapy.[61] The FIRE-4 and PARADIGM studies demonstrated that mCRC patients with *RAS*–wild type disease based on tissue but were pretreatment plasma positive for *BRAF*/*RAS* mutations had worse outcomes when treated with a regimen containing anti-EGFR therapy.[62,63] These results collectively raise the possibility that plasma, in conjunction with tissue testing, can better capture intratumor and intertumor heterogeneity and resistant subclones, and identify potential treatment unresponsiveness.[63]

In gastroesophageal cancer, retrospective studies and post-hoc analyses from interventional trials suggest actionable alterations, such as *HER2* and *EGFR* amplifications, identified in the plasma and/or tissue can predict responses to targeted therapy.[29] ctDNA was also used to identify resistance mechanisms through the loss of mutated clones or upregulation of bypass pathways after exposure to HER2- or EGFR-directed therapies.[29]

In PDAC, a retrospective study of multigene ctDNA testing in 282 patients identified potentially actionable alterations in 48% of patients, with rates of targetable homologous recombination gene mutations and *KRAS* G12C mutations reported as 8.8% and 2.6%, respectively.[46] Another study identified a 29% incidence of potentially targetable gene alterations using a 60-gene panel in 48 patients with metastatic PDAC.[64] Large-scale efforts are underway to molecularly characterize HCC using ctDNA to identify prognostic and targetable alterations.[40,49]

Although ctDNA data are promising, there is no standardization on which assay to use in clinical practice. Predictive biomarker data are largely from retrospective, exploratory analyses in small numbers of patients. Accounting for the sensitivity limitations of ctDNA assays and variable ctDNA shedding rates across tumors, the absence of an actionable alteration in the plasma

should be interpreted with caution and should prompt reflex tissue testing. However, if tissue biopsy is not feasible or samples are exhausted, ctDNA can be considered for blood-based NGS. This recommendation is supported by recommendations from the National Comprehensive Cancer Network and the European Society for Medical Oncology.

Applications in Screening and Diagnosis

The Epi proColon test (Epigenomics) is the only FDA-approved blood-based screening test for GI cancers. This test screens for CRC by detecting circulating methylated SEPT9 DNA. It has a sensitivity of 68.2% to 73.3% and a specificity of 78.8% to 81.5% when using colonoscopy as a reference standard, and has better sensitivity but significantly worse specificity when compared with the fecal immunochemical test.[65] A positive result should be followed up with a diagnostic colonoscopy. Other liquid biopsy tests are also under investigation for CRC screening (NCT04136002, NCT04369053, and NCT05875584).

Several ctDNA assays are being studied for broad cancer screening and tumor origin testing. CancerSEEK is a 61-amplicon panel that detects 16 genes and 8 protein biomarkers to detect cancer. Cohen and colleagues used CancerSEEK on 1005 patients with untreated early-stage cancers and 812 healthy controls. The test had 70% sensitivity and 99% specificity; however, sensitivity was lower for earlier-stage (stage 1) disease. Tumor origin testing successfully localized the tumor to a single site in 63% of cases and to 2 potential sites in 83% of cases.[50] Validation of their classification algorithm is being studied in the ASCEND trial (NCT04213326). In DETECT-A, which included 9941 participants, 26 cancers were detected with CancerSEEK and positron emission tomography imaging, 24 were detected with SOC approaches, and 46 were not detected by either.[66]

The CCGA study used whole-genome bisulfite sequencing to detect methylation patterns in cfDNA to screen for cancer. The test specificity was 99.3%, with 93% accuracy in determining the tissue of origin. However, sensitivities varied widely by stage and were as low as 18% for stage 1 to as high as 93% for stage 4.[67] In PATHFINDER, another methylation-based cfDNA assay called Galleri (Grail) also demonstrated promising screening performance and tumor-localizing performance.[68] The ongoing PATHFINDER 2 study is using Galleri with a more targeted diagnostic approach based on tumor origin signals (NCT05155605).

Many ongoing trials are evaluating other liquid biopsies in cancer screening (NCT05099068, NCT05516927, NCT05516927, and NCT05227261). Today, the sensitivities of these assays are limiting, especially in earlier stages where early detection and subsequent treatments are assumed surrogates for survival. A standardized diagnostic workflow after a positive screen has yet to be established, and the survival benefit and cost-benefit analysis for these liquid assays in population-wide screenings are unknown.

Limitations

With the increasing availability of commercial ctDNA tests, providers must be aware of their limitations. Studies have used variable assays; quantification thresholds across genes; and testing intervals, with or without landmark analyses. In the absence of any standardization, the clinical significance and application of results should always be interpreted with reservation. Caution is also advised when making interstudy comparisons given the heterogeneity in study designs, even when studies are using the same assay.

Tumor-informed and tumor-agnostic assays have unique strengths and limitations, but the decision to use one over the other should be based on the clinical question. Many assays cannot detect gene deletions or complex rearrangements, may be limited in sensitivity, and are liable to false positives when confounded with clonal hematopoiesis of indeterminate potential (CHIP).[69] Some limitations may be circumvented by technological refinement to improve sensitivity/specificity, serial testing, utilizing other clinical diagnostic tools, and implementing white blood cell control or methylated approaches in the case of CHIP.[69]

ctDNA detection is also subject to biological factors that influence tumor shedding, including histology, burden, intratumor/intertumor heterogeneity, and anatomic sites of disease.[33,69] Therefore, negative ctDNA results should be interpreted with caution, considering that ctDNA levels may be below a detection threshold or that a tumor is a "poor shedder." Further validation studies are needed before ctDNA is routinely used in cancer detection/screening, especially for tumors without screening guidelines but have high mortality rates, and for treatment escalation and deescalation.

Circulating Tumor Cells

CTCs can be detected in the peripheral blood and leveraged to study cancer metastases. Several platforms, each using different methods, have been developed to isolate CTCs. CellSearch (Menarini) is an antibody-dependent assay that isolates epithelial cell adhesion molecule–positive, CD45-negative cells. It is FDA-approved as a prognostic marker for CRC and is actively being studied in other GI cancers.[70,71] Other platforms include Parsortix (Angle), a cell size–based assay that can capture both epithelial and mesenchymal tumor cells, and cell-surface biomarker assay from RareCyte that utilizes immunofluorescent staining to select out CTCs. Both have shown promising applications in the GI cancer space.[72]

Studies have demonstrated that patients with mCRC

Table 2. Prospective ctDNA-Based Therapeutic and Surveillance Intervention Trials in the MRD Setting for Other GI Cancers

Trial, phase	Patient population	Design	Intervention	Primary endpoints	Secondary endpoints
NCT05482516 (MRD-GI), 3	CRC, HCC, gastric cancers, and PDAC with ctDNA+ status/NED on scans after all SOC curative-intent interventions	Single arm	Atezolizumab + bevacizumab	Rate of Signatera ctDNA+ Rate of enrollment 12 mo Rate of ctDNA CR 12 wk Rate of ctDNA PR 12 wk Rate of ctDNA POD or relapse 12 wk	Toxicity, reasons for failure of enrollment
NCT05788744 (CIRCPAC)	PDAC after resection	Randomized	Arm 1: ctDNA every 3 mo. ctDNA+ will have CT scan, EUS surveillance. ctDNA– will have CT scan and EUS every 6 mo Arm 2: SOC surveillance	Preoperative ctDNA and eccDNA predicting recurrence DFS OS Rate of recurrence assessed by eccDNA	
NCT05638698 (TESLA), 2	PDAC after resection with ctDNA+	Randomized	Randomized to: TG01 (vaccine)/QS-21 (drug to improve TG01 response) TG01/QS-21 + balstilimab	6-mo ctDNA control rate	TRAEs, DFS, CMR rate, correlation of CMR with DFS
NCT04853017 (AMPLIFY-201), 1	*KRAS*-mut PDAC following surgery or chemoradiation with ctDNA+ or positive serum markers	Sequential assignment	ELI-002 2P (Amph-modified *KRAS* peptides) vaccine	MTD, safety	ctDNA reduction/ clearance rate
NCT05802407 (MAP-02)	PDAC following resection with ctDNA rise after adjuvant therapy but NED radiographically	Randomized	Randomized to: Switching to a later-line therapy Continuing current therapy	DFS	MRD prognosis, prognostic role of MRD-guided therapy

ctDNA, circulating tumor DNA; CMR, complete metabolic response; CR, complete response; CRC, colorectal cancer; DFS, disease-free survival; eccDNA, extrachromosomal circular DNA; EUS, endoscopic ultrasound; HCC, hepatocellular carcinoma; MRD, measurable residual disease; mo, months; MTD, maximum tolerated dose; NED, no evidence of disease; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; PFS, progression-free survival; POD, progression of disease; PR, partial response; SOC, standard of care; TRAEs, treatment-relate adverse events; wk, week(s).

and baseline pretreatment and longitudinal blood draws with more than 3 CTCs per 7.5 mL of blood have worse survival than patients with less than 3 CTCs per 7.5 mL of blood.[73,74] Other studies using various testing platforms similarly demonstrate the association between CTC detection and quantification with stage and survival.[71,73,75] Similar prognostic trends, although with variable CTC cut-offs, are also reported in patients with other GI cancers. In upper GI cancers, a pretreatment CTC count of 2 or greater predicted worse survival, as did the change in CTC count with treatment.[76] In a 2019 meta-analysis of more than 4000 patients with gastric cancer, patients with detectable CTCs had worse OS than those with undetectable CTCs (HR, 1.84; 95% CI, 1.50-2.26; P<.001).[77] A 2014 meta-analysis including 623 PDAC patients reported similar trends.[78] Other studies have reported the prognostic value of CTC cut-offs of 2 and 5 in advanced cholangiocarcinoma patients and the prognostic value of CTC positivity in patients with HCC.[79,80]

Studies have also demonstrated the prognostic value of CTCs in the perioperative setting across different GI tumors.[81-84] Other studies have shown CTC surface markers and DNA/RNA can be analyzed for molecular alterations (eg, *FGFR2* overexpression in gastroesophageal cancer) and serve as another, less-invasive method to

identify actionable mutations.[84] Another exciting frontier in CTC research now involves propagating an individual patient's CTCs to create xenograft or organoid models to study the efficacies of therapeutics ex vivo, with direct translational clinical implications.[10,11]

CTCs are currently harder to detect than ctDNA, and the assays are costly. CTCs are present at lower levels (<20 cells/10 mL of blood), are fragile, and have nonspecific cell surface markers, making them more difficult to isolate.[5] Furthermore, propagating CTCs reliably for research is only successful in 6% to 20% of cultures. Other novel platforms show promise and have the potential to develop more reliable CTC cultures, xenograft models, and biomarker detection.[11,71,72,80] With ongoing optimization, CTCs have the potential to serve as a multipotent clinical biomarker and research tool to study tumor characteristics down to a single cell level and help facilitate drug development in a way that can augment or even surpass ctDNA.

RNA, Exosomes, Proteins, and Metabolomics

Other non-ctDNA liquid biopsies are increasingly being explored in GI malignancies. Extracellular RNA (exRNA) assays may have higher sensitivities and specificities than ctDNA assays, given their resistance to degradation and tissue-specific expression. Several single-RNA biomarkers and RNA panels that may aid in diagnosis and prognosis have been identified. A few examples include a 7-microRNA (miRNA) panel that can distinguish early-stage esophageal cancer from the absence of cancer with a sensitivity of 89.6% and specificity of 79%; an 8-miRNA panel to detect early-stage HCC in at-risk individuals with 97.7% sensitivity and 94.7% specificity; and miR-4772-3p upregulation being associated with shorter time to recurrence in CRC.[85,86]

Tumor exosomes, with enhanced stability and inclusion of multiple cellular components (lipids, proteins, RNA), may offer advantages over ctDNA. Examples of promising markers include glypican-1, a potential screener for early PDAC, and CXCL7, which could be a response marker for mCRC with liver metastasis.[87,88] Blood assays leveraging cfDNA fragmentomics and cancer metabolomics are also showing promise for the detection and localization of GI cancers.[12,89-93] Although these liquid biopsies are still in their infancy in development, they hold tremendous prospects.

Alternative Forms of Liquid Biopsy

Liquid biopsies in other fluid compartments outside of the plasma have also been studied but to a lesser extent. In a study of 304 GI cancer patients with known peritoneal carcinomatosis, higher ctDNA allele frequencies were found in the peritoneal fluid than in plasma (50% vs 3%; $P<.0001$). Furthermore, patients with peritoneal-only metastasis had lower plasma ctDNA allele frequencies compared to patients with metastases to distant organs (1% vs 5.6%; $P<.0001$).[94] These findings suggest that patients with peritoneal-only disease may shed relatively less ctDNA in the plasma; however, higher levels can be seen in the peritoneal fluid, which could theoretically be used to diagnose peritoneal disease and perform genomic analysis if tissue is otherwise limited. Other studies suggest peritoneal ctDNA after hyperthermic intraperitoneal chemotherapy is prognostic for recurrence.[95]

Urine cfDNA methylation assays have the potential to screen for malignancies.[96] Additionally, urine cfDNA can be prognostic and predictive in GI tumors, but these studies are limited in number, with small patient samples.[97] Other studies suggest that cerebrospinal fluid cfDNA analysis may be more sensitive than cytology in diagnosing leptomeningeal disease.[98] These alternatives appear to have a good diagnostic yield in GI malignancies, especially in scenarios where imaging, plasma ctDNA, or fluid cytology are otherwise uninformative. However, more studies are needed in this space.

Conclusion

Liquid biopsy has emerged as an exciting new frontier in oncology, with the potential to personalize treatment strategies and optimize patient outcomes. Although ctDNA testing is the farthest along in research and is now adopted in clinical practice, its limitations and need for further validation as a predictive biomarker must be acknowledged. On the horizon, CTCs, exosomes, proteomics, and metabolomic studies are underway. With the understanding that optimization of liquid biopsy techniques and ongoing collaborative research efforts are vital to raising the bar, we anticipate that liquid biopsies will increasingly become an integral tool in cancer management.

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