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Use of Circulating Cell-Free DNA to Guide Precision Medicine in Patients with Colorectal Cancer

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Abstract

Patient-specific biomarkers form the foundation of precision medicine strategies. To realize the promise of precision medicine in patients with colorectal cancer (CRC), access to cost-effective, convenient, and safe assays is critical. Improvements in diagnostic technology have enabled ultrasensitive and specific assays to identify cell-free DNA (cfDNA) from a routine blood draw. Clinicians are already employing these minimally invasive assays to identify drivers of therapeutic resistance and measure genomic heterogeneity, particularly when tumor tissue is difficult to access or serial sampling is necessary. As cfDNA diagnostic technology continues to improve, more innovative applications are anticipated. In this review, we focus on four clinical applications for cfDNA analysis in the management of CRC: detecting minimal residual disease, monitoring treatment response in the metastatic setting, identifying drivers of treatment sensitivity and resistance, and guiding therapeutic strategies to overcome resistance.

INTRODUCTION

Next-generation sequencing (NGS): A high-throughput testing platform that enables simultaneous sequencing of the base pairs in DNA or RNA samples. Applications include gene expression profiling, detection of epigenetic changes, and molecular analyses

Interlesional heterogeneity: Genomic differences between different tumor lesions in the body

Intralesional heterogeneity: Genomic differences between tumor cells in the same lesion

Circulating cell-free DNA (cfDNA): DNA in the bloodstream or other body fluid, including germline DNA and mutated DNA

Circulating tumor DNA (ctDNA): 150–200-base-pair fragments of DNA that originate from cancer cells and are present in the bloodstream or other body fluid

Prognostic biomarker: A predictor of clinical outcome independent of specific treatment effects; distinct from a predictive biomarker, which can be used to identify patients who are most or least likely to respond to a given therapy

Precision medicine has driven survival breakthroughs in the management of metastatic colorectal cancer (CRC). Molecular diagnostics—such as next-generation sequencing (NGS) of tumor tissue—enable precision medicine by allowing simultaneous interrogation of multiple genomic targets and predicting treatment response. Despite the clinical utility of these advanced molecular diagnostics, nearly half of all cancer patients in the United States are not receiving guideline-based molecular testing (1). Availability of tumor tissue is one of the primary barriers to molecular testing (2). Even when tumor tissue is available, heterogeneity between lesions (interlesional heterogeneity) and within the same lesion (intralesional heterogeneity) may limit detection of clinically relevant alterations (3–6). Additionally, tumors may evolve under the selective pressure of therapy, and recognition of such changes requires serial biopsies (7). Given the additional cost and risk of invasive tumor tissue biopsies, serial monitoring of tumor tissue is impractical. In order to fulfill the potential of precision medicine, noninvasive and cost-effective molecular testing strategies are needed.

Circulating cell-free DNA (cfDNA) is shed from cells throughout the body and is detectable from a routine blood draw. Additionally, as tumor tissue undergoes apoptosis and necrosis, fragments of tumor-derived DNA are released into the bloodstream. The portion of cfDNA derived from tumor tissue is defined as circulating tumor DNA (ctDNA). Approximately 85% of patients with metastatic CRC have detectable ctDNA (8, 9). In addition to ctDNA, tumor tissue may release other cellular components into the bloodstream, including cell-free RNA, proteins, and exosomes. These cellular components are detectable in other body fluids, such as urine, stool, pleural fluid, and ascites (10–12). Given the widespread availability of commercial assays for clinical use, this review focuses on the use of blood-based cfDNA.

Compared to molecular profiling of tumor tissue, analysis of cfDNA offers several advantages. Analysis of cfDNA requires a routine blood draw, which is both safer and more convenient than an invasive tumor tissue biopsy. Additionally, compared to tumor tissue profiling, cfDNA profiling is generally less resource intensive and has a rapid turnaround time (13, 14). Finally, the ease and convenience of cfDNA collection allows serial monitoring of emergent alterations, particularly acquired resistance alterations that are not present in all metastatic lesions (7, 15, 16). Multiple studies and meta-analyses have established ctDNA as a prognostic biomarker, with higher levels of ctDNA corresponding to decreased survival (17). Nonetheless, questions remain regarding concordance between tissue- and blood-based molecular assays (18, 19). These studies highlight the limitations of cfDNA assays as a diagnostic tool, but they also demonstrate how best to apply this emerging technology. Since cfDNA assays require ctDNA shedding into the bloodstream, the performance of cfDNA assays is improved when blood is collected after—rather than during—active chemotherapy. Additionally, concordance is optimized when ctDNA shedding is high—either due to high absolute tumor burden or highly vascularized metastatic lesions. Recent large-scale analyses of patients with metastatic CRC have demonstrated strong concordance between tissue- and plasma-based analyses (20, 21). Collectively, these studies support the clinical validity of cfDNA assays and support prospective studies to demonstrate clinical utility.

Several technologies have emerged in recent years to detect ctDNA. Different assays offer varying levels of sensitivity, specificity, cost, and clinical utility. Polymerase chain reaction (PCR)-based methods, including droplet digital PCR (ddPCR) and BEAMing (beads, emulsion, amplification, and magnetics), offer high specificity and sensitivity (22, 23). Of those assays, ddPCR is optimized for rapid detection of simple genomic alterations, such as substitution mutations (14). By comparison, NGS platforms offer analysis of multiple genomic targets and alterations. Although NGS technology is generally more expensive and has a longer turnaround time than ddPCR, it

is optimized to monitor clonal evolution of acquired resistance mutations (24). Patient-specific tumor-informed panels offer high sensitivity and specificity, but they can have long turnaround times and require knowledge of the genomic target in tissue before performing blood-based testing. The tumor-informed approach is particularly well suited for detection of minimal residual disease (MRD), which requires ultrasensitive and highly specific assays (25–27). In summary, the optimal ctDNA assay should be fit for purpose and optimized for the intended clinical application. A more in-depth review of diagnostic technologies is available elsewhere (11, 28, 29).

In this review, we present four clinical applications of cfDNA to guide precision medicine in patients with CRC (**Figure 1**): detecting MRD following surgery, monitoring treatment response in the metastatic setting, identifying genomic drivers of therapeutic sensitivity and resistance, and guiding treatment strategies to overcome therapeutic resistance.

Droplet digital PCR (ddPCR): Diagnostic technology that combines digital PCR with water–oil emulsion droplet technology. A sample is fractionated into microscopic droplets, and PCR amplification of the template molecules occurs in each individual droplet

BEAMing (beads, emulsion, amplification, and magnetics): Diagnostic technology that combines digital PCR with magnetic beads and flow cytometry to provide ultrasensitive detection of mutated DNA fragments

Tumor-informed: A tumor-informed ctDNA analysis is custom designed to detect alterations identified from the patient's tumor tissue

Minimal residual disease (MRD): Evidence for residual or recurrent malignancy in the absence of radiographic or biochemical relapse

DETECT MINIMAL RESIDUAL DISEASE

After surgery with curative intent, the goal of adjuvant chemotherapy in stage II/III CRC is to eradicate remnant micrometastatic disease (30–32). Adjuvant chemotherapy reduces the risk of recurrent disease, but it has toxicity and does not benefit all patients. Patients who were already cured by surgery alone are overtreated with adjuvant chemotherapy, while other patients are inappropriately deemed low risk and are potentially undertreated. The inability to identify risk of recurrence—beyond conventional surgical staging—complicates adjuvant treatment decision making and potentially impairs patient survival. Some molecular assays can identify patients who are at high risk for recurrence, but these assays have thus far failed to demonstrate that adjuvant chemotherapy alters the outcome (33–35). There remains an unmet need for a clinically validated assay that can inform clinicians which patients with resected CRC will benefit from adjuvant chemotherapy.

For patients with solid tumors, detection of ctDNA following definitive therapy is a highly sensitive and specific method for identification of MRD (36–38). Technological advancements have allowed detection of ctDNA variant allele fractions as low as <0.01% (39). These ultrasensitive assays can detect extremely rare mutated DNA fragments in a sea of germline DNA. To incorporate these assays into clinical studies and ultimately into clinical practice, assay design and performance are crucial. Incorporation of higher numbers of tumor-specific probes—which expands the number of variants across the tumor genome able to be captured—increases the sensitivity further, with sensitivities exceeding 75% (8, 40–42).

Across all stages of resected CRC, detection of ctDNA following surgery is a strong predictor of disease relapse. Tie et al. (26) sequenced tumors from >200 patients with stage II colon cancer, using a panel of 15 of the most commonly mutated genes in this cancer type. Mutations were detected in >99% of sequenced tumors. The most prevalent mutation was then selected for design of a personalized ctDNA probe. Among 178 of these patients with stage II colon cancer who received no adjuvant chemotherapy, ctDNA was detected postoperatively in 8% of cases. All patients who had ctDNA detected following surgery experienced a recurrence within 24 months. In contrast, three-year relapse-free survival for patients without detectable postoperative ctDNA was >90%. This study highlights the value of tumor-informed postoperative ctDNA as a predictor of recurrence, and it suggests that ctDNA status outperforms all traditional high-risk factors for stage II colon cancer (e.g., tumor differentiation, T3 versus T4 stage, number of lymph nodes dissected, perforation, lymphovascular invasion). In addition to predicting recurrence, ctDNA status outperforms current standard-of-care surveillance tools. Scholer et al. (43) reported that ctDNA detection preceded radiographic evidence of recurrence, with a median lead time of 9.4 months. Additionally, ctDNA status detects recurrence more than 3 months earlier than carcinoembryonic

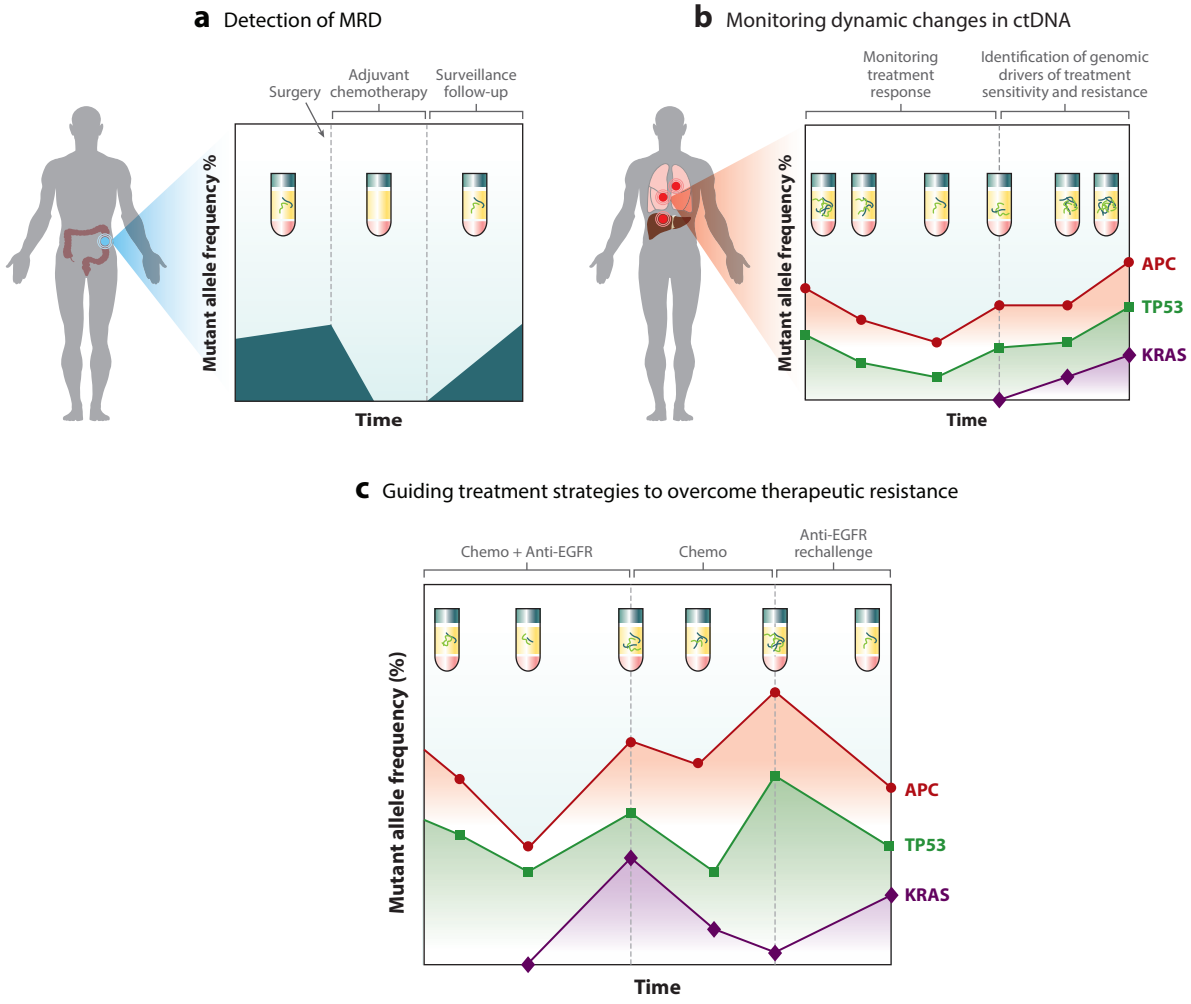

Figure 1

Clinical applications of cell-free DNA (cfDNA) in patients with colorectal cancer. (*a*) Detection of minimal residual disease (MRD). Following resection of the colorectal primary, tumor tissue is sequenced, and a personalized blood-based cfDNA assay is created to monitor for recurrence. Blood is then drawn serially, and cfDNA is analyzed for circulating tumor DNA (ctDNA) variants specific to the colorectal tumor. (*b*) Monitoring dynamic changes in circulating tumor DNA (ctDNA). In the metastatic setting, palliative therapies are designed to provide disease control and maintain quality of life. Mutations specific to the tumor can be followed serially to identify response or resistance to therapy. New tumor mutations may emerge on treatment, and these can drive treatment resistance and be detected in cfDNA. (*c*) Guiding treatment strategies to overcome therapeutic resistance. Mutations in the MAP kinase signaling pathway (*KRAS*, *NRAS*, *BRAF*, *MAP2K1*) and amplification of receptor tyrosine kinases may drive anti-EGFR (epidermal growth factor receptor) resistance. These genomic alterations can be detected in blood either before or after the start of anti-EGFR therapy. Genomic alterations that emerge on treatment with anti-EGFR therapies will decay and disappear after treatment is discontinued. Analysis of cfDNA reveals whether these acquired genomic alterations have disappeared and whether the patient might be appropriate for EGFR rechallenge.

antigen (CEA)—a standard blood-based tumor marker. Collectively, current evidence establishes ctDNA assays as a more sensitive modality for detection of low-volume recurrent disease and supports prospective studies to validate ctDNA status as a standard-of-care survivorship test.

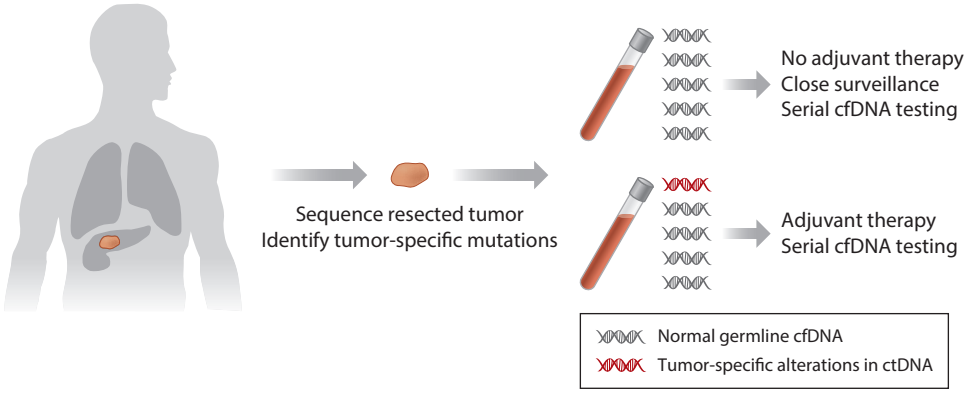

Figure 2

Tumor-informed cell-free DNA (cfDNA) profiling to guide adjuvant chemotherapy decision making. First, the resected tumor is sequenced to identify tumor-specific mutations representing a unique mutational signature for each individual patient. Next, cfDNA is tested for the residual presence of these tumor-specific mutations using highly sensitive and specific methods. Detection of any tumor-specific mutations in cfDNA at this postoperative time point would provide direct evidence of residual tumor, and this information could be used to guide adjuvant treatment decisions. Figure reproduced with permission from Reference 45.

In addition to determining who needs adjuvant chemotherapy, it is possible that ctDNA assays will also guide adjuvant treatment intensity and duration (**Figure 2**). While current guidelines advise adjuvant chemotherapy for all patients with stage III CRC, there has been increased acceptance of a shorter duration of combined cytotoxic treatments for lower-risk patients (44). A potential application of ctDNA status is de-escalation of adjuvant chemotherapy in low-risk ctDNA$^-$ patients who have no residual disease after surgery. The utility of ctDNA status in decisions to de-escalate adjuvant chemotherapy (reduce duration or intensity of chemotherapy) in ctDNA$^-$ patients and intensify adjuvant chemotherapy (restart or utilize novel therapies) in ctDNA$^+$ patients is an area of future research (see sidebar titled Surgical Decision Making for Locally Advanced Rectal Cancer: Is There a Role for ctDNA?).

Despite the promise of ctDNA for MRD detection, challenges remain. Although ctDNA is a validated prognostic biomarker (25, 46–48), prospective studies have not yet demonstrated that

SURGICAL DECISION MAKING FOR LOCALLY ADVANCED RECTAL CANCER: IS THERE A ROLE FOR ctDNA?

Current management of locally advanced (clinical stage II/III) rectal cancer includes neoadjuvant chemoradiation, and in some centers, chemotherapy is given prior to surgery (total neoadjuvant therapy). When patients experience complete response to treatment, it is unknown if surgical resection is necessary. For these patients with complete clinical response, ctDNA may have a future role to guide surgical decision making. A study of patients with locally advanced rectal cancer found no detectable ctDNA in 20 of 21 patients (95%) who achieved a complete pathologic response with neoadjuvant chemoradiation (97). At the same time, ctDNA was undetectable in 80 of 91 (88%) patients with a noncomplete pathologic response after receiving the same treatment. Whether or not ctDNA status can identify patients who are more appropriate for a watch-and-wait approach that forgoes surgical resection will be investigated in future clinical trials. Pending results from these upcoming trials, ctDNA testing should not be incorporated into routine clinical management of locally advanced rectal cancer.

ctDNA status can be used to alter patient outcomes. Several studies have evaluated whether adjuvant chemotherapy can eliminate ctDNA. In a small series of 18 patients with stage III colon cancer receiving adjuvant chemotherapy, ctDNA status seroconverted to nondetectable in 9 cases (50%) (46). The patients who cleared ctDNA had reduced recurrence risk compared to those with persistent ctDNA. Similarly, another series reported a 30% clearance rate of ctDNA with adjuvant chemotherapy (25). Those patients who remained ctDNA$^+$ following adjuvant chemotherapy had significantly greater risk of recurrence. Although these case series have shown that it is possible to eliminate ctDNA with adjuvant chemotherapy, limited prospective evidence exists to support a survival benefit. Future studies will need to demonstrate improved survival for the prognostically poor ctDNA$^+$ patient population. Therapeutic strategies may include additional cytotoxic agents or molecularly targeted/matched biologic agents, especially since these same ctDNA assays potentially identify actionable mutations.

Epigenomic profiling: The study of methylation of cellular DNA or modification of histones that alter gene expression without changing the DNA sequence

Truncal mutations: Genomic alterations that appear early in cancer development and are present in all tumor cells throughout the natural history of the disease

Complementary diagnostic strategies may enhance the sensitivity of cfDNA assays to detect recurrent disease. Malignant tumors feature differentially methylated regions that are distinct from adjacent nonmalignant tissue (49, 50). For example, methylation of *IKZF1* and *BCAT1* is more likely in CRC tumor tissue than in nonmalignant tissue (51). In a cohort of 138 patients with resected CRC, methylation of *IKZF1* and *BCAT1* was associated with a higher likelihood of recurrence (52). Epigenomic profiling may play a future role in enhancing the sensitivity of ctDNA assays and detecting occult disease.

MONITOR TREATMENT RESPONSE

In the metastatic setting, serial quantitative analysis of ctDNA is a powerful tool to monitor treatment response (53, 54). In general, absolute ctDNA levels directly correlate with total tumor burden. This linkage intuitively implies that higher numbers of cancer cells generate more ctDNA. Additionally, higher pretreatment ctDNA levels have been associated with poorer cancer-specific and overall survival outcomes (55, 56). However, the association between total ctDNA and survival is impacted by the pattern of metastatic spread, as disease confined to the peritoneum or lungs has lower rates of ctDNA shedding than liver or bone disease (57). Therefore, application of this technology in monitoring treatment response must be considered in the context of an individual's clinical presentation.

Truncal mutations—which appear early in cancer development and persist throughout the treatment course—are ideally suited for tracking treatment response. For example, *BRAF*^{V600E} mutations are present in approximately 10% of patients with metastatic CRC (58) and appear even in precancerous lesions, developing along the sessile serrated pathway (59). For assessing treatment response in such patients, tracking *BRAF*^{V600E} levels may be a surrogate for imaging or tumor marker (CEA) measurement. In a phase I trial of vemurafenib, cetuximab, and irinotecan in patients with *BRAF*^{V600E} mutated metastatic CRC, all patients who experienced a partial or complete radiographic response had a >90% drop from baseline *BRAF*^{V600E} ctDNA levels (60). Similarly, in another study of patients with *BRAF*^{V600E} metastatic CRC treated with a combination of BRAF, EGFR, and MEK inhibitors, log-fold changes in *BRAF*^{V600E} ctDNA levels outperformed CEA changes in predicting early radiographic treatment response (61). Therefore, change in truncal mutations in ctDNA is a promising tool to identify treatment response.

More expansive gene panels may enhance the sensitivity of ctDNA to monitor treatment response. In a series of 94 patients with metastatic CRC tested with a 54-gene or 73-gene NGS cfDNA panel, the median number of mutations detected in the plasma was three (41). Here, the most common mutations were in *TP53*, *APC*, and *KRAS*—genes known to be among the most commonly mutated in patients with CRC (62). In this cohort, nearly 80% of these patients had at least one alteration reported in ctDNA that could be used in order to track responses serially.

For patients with incurable CRC, ctDNA assays can predict an earlier response (or lack thereof) across the landscape of systemic therapies, which may aid in refining personalized treatment approaches. For example, one group looked at serial ctDNA levels over time in 53 patients with CRC receiving standard chemotherapy to assess the impact of ctDNA change on treatment response (63). The mutated gene with the highest variant allele fraction was followed prior to treatment, at day 3, and prior to initiation of cycle 2. Depth of reduction of ctDNA levels was associated with radiographic response. Patients experiencing tenfold drops in absolute ctDNA levels demonstrated longer progression-free survival (PFS) than those with less substantial reductions. Of note, ctDNA changes at day 3 did not correlate with clinical outcome. This observation suggests that timing of ctDNA assessment is critical to assay performance.

At present, the optimal timing for ctDNA assessment has not been validated and may depend on the specific type of therapy and methodology of ctDNA measurement. In a retrospective study of 101 patients with advanced gastrointestinal malignancies receiving standard cytotoxic agents (83%) and/or targeted therapies (30%), ctDNA change measured at 4 weeks—but not at 2 weeks—correlated with radiographic response and survival benefit (64). Notably, the study included patients with diverse treatment histories and tumor types, but the majority of enrolled patients had refractory CRC. Patients whose ctDNA levels did not drop by at least 30% after 4 weeks of treatment were significantly less likely to benefit from therapy. Meanwhile, in a separate cohort of patients with treatment-refractory metastatic CRC receiving regorafenib, an early rise in ctDNA levels at 14 days correlated with worse PFS and survival (55). The optimal timing for ctDNA assessment requires further clinical validation.

Collectively, initial studies in patients with metastatic CRC demonstrate the potential clinical utility of ctDNA assessment to identify early treatment response. To minimize the toxicity of futile therapy, patients with unfavorable ctDNA change may warrant early therapeutic switch. Future prospective studies will be critical to validating ctDNA as a diagnostic tool for monitoring treatment response and optimizing palliative therapies.

Secondary resistance: Therapeutic resistance after an initial period of disease stabilization or response

Primary resistance: Radiographic or clinical disease progression as the best response to an anticancer therapy

Subclonal: A subclonal genomic alteration is present in a minority of tumor cells. These alterations may increase or decrease relative to truncal alterations under treatment pressure

Variant allele frequency: The relative frequency of a mutated allele compared to all alleles in the population

IDENTIFY GENOMIC DRIVERS OF TREATMENT SENSITIVITY AND RESISTANCE

The goal of targeted cancer therapies is to provide potent antitumor activity while minimizing treatment-related side effects. Unfortunately, most patients eventually develop therapeutic resistance. Genomic drivers of resistance exist as rare subclones prior to the initiation of targeted therapy, but proliferate relative to sensitive clones under the selective pressure of therapy (65, 66). Compared to tumor tissue biopsies, the liquid biopsy is particularly well suited to identify emergent genomic drivers of resistance, particularly when they exist in a background of interlesional and intralesional heterogeneity (67). Additionally, by serial monitoring of emergent resistance alterations, cfDNA analysis can predict the time to treatment failure (6, 65, 66, 68).

In multiple tumor types, cfDNA can identify acquired resistance (secondary resistance) alterations. In many cases, the impact of these secondary resistance alterations on clinical decision making and therapy selection is significant (69, 70). In patients with metastatic CRC, cfDNA has been particularly effective at identifying genomic drivers of resistance to cetuximab and panitumumab—two standard-of-care EGFR monoclonal antibodies. Mutations in the mitogen-activated protein kinase (MAPK) signaling cascade—including *BRAF*, *KRAS*, and *NRAS*—drive primary and secondary resistance to anti-EGFR antibodies (71–73). In some cases, a MAPK pathway mutation is present in blood at a low (subclonal) variant allele frequency prior to the initiation of anti-EGFR therapy and then expands relative to wild-type clones under treatment pressure (6). In PROSPECT-C, a single-arm phase II trial of cetuximab in *RAS* (*KRAS* and *NRAS*) wild-type metastatic CRC, patients with *RAS*, *BRAF*, or *PIK3CA* mutations detected in pretreatment cfDNA

had shorter PFS than patients without these alterations (hazard ratio 3.41; 95% confidence interval 1.24–9.37; $p = 0.02$). The results from PROSPECT-C highlight the limitations of tumor tissue profiling to identify subclonal *RAS* mutations and predict treatment benefit.

In patients with *RAS* wild-type metastatic CRC receiving anti-EGFR therapies, *RAS* mutations are the most common alterations detected in blood at progression (66, 67, 74, 75). In comparison to *KRAS* exon 2 mutations, which have the greatest prevalence in EGFR treatment-naïve patients, acquired *RAS* mutations are more likely to occur in exons 3 and 4 of *KRAS* and *NRAS* (7, 76). In addition to *RAS* mutations, mutations in *MAP2K1* and *BRAF* are known to emerge under the selective pressure of treatment—often together with *RAS* mutations—and drive acquired resistance to anti-EGFR therapy (7, 9).

In addition to mutations in the MAPK pathway, *EGFR* ectodomain (ECD) mutations drive resistance to anti-EGFR therapies. *EGFR* ECD mutations alter the binding domain of cetuximab and panitumumab, thereby preventing receptor engagement (77). In some cases, the *EGFR* ECD mutation is specific to only one EGFR antibody. For example, $EGFR^{S492R}$ prevents cetuximab binding but does not alter panitumumab binding. A randomized trial of cetuximab versus panitumumab in patients with refractory metastatic CRC found an $EGFR^{S492R}$ ECD mutation in the cfDNA of 16% of patients treated with cetuximab, compared to only 1% of patients treated with panitumumab (78). Other *EGFR* ECD mutations—including R451C, K467T, S464L, G465R, and I491M—are also detected in cfDNA and are known to drive anti-EGFR resistance (79). *EGFR* ECD mutations are associated with prolonged exposure to EGFR antibodies and have a more favorable prognosis than acquired *RAS* mutations (74).

Molecular profiling of cfDNA has identified additional drivers of anti-EGFR resistance. *MET* amplification—which activates the MAPK and phosphoinositide 3-kinase (PI3K) signaling pathways—is found in approximately 1–2% of CRC tumors prior to anti-EGFR therapy and is associated with primary anti-EGFR resistance (80, 81). In a single-institution study in which cfDNA profiling was performed on 205 patients with *RAS* wild-type metastatic CRC, *MET* amplification was detected in 4.7% of patients prior to anti-EGFR therapy compared to 22.6% of patients after progression on anti-EGFR therapy ($p < 0.001$) (82). In addition to *MET* amplification, *HER2* (*ERBB2*) amplification is detectable in blood and is associated with primary and acquired anti-EGFR resistance (83–85). In a study of nine patients with anti-EGFR-resistant *RAS* wild-type metastatic CRC, *HER2* amplification was detected in four of nine post-EGFR plasma samples. Three of these patients had nonamplified tumors prior to anti-EGFR treatment but developed *HER2* amplification at progression. In two of these three cases, the conversion to *HER2* amplification was detected in tissue and blood (85). Finally, other rare drivers of anti-EGFR resistance—including *KRAS* amplification—can be detected in cfDNA and are enriched after progression on anti-EGFR therapies (9, 86). The use of cfDNA has identified several genomic drivers of anti-EGFR resistance and inspired innovative therapeutic strategies.

SUPPORT THERAPEUTIC STRATEGIES TO OVERCOME RESISTANCE

Most patients with *RAS* wild-type metastatic CRC who progress on anti-EGFR therapy have one or more resistance alterations detected in blood (9, 74, 75). Importantly, postprogression cfDNA reveals significantly greater molecular heterogeneity than tumor tissue. Parikh et al. (40) reported molecular profiling results from a cohort of 23 patients who received simultaneous blood collection and a tumor tissue biopsy after progression on targeted therapy. The use of cfDNA detected at least one resistance alteration in 87% of patients compared to only 48% of patients using tumor tissue. In a separate cohort of 42 patients with metastatic CRC, cfDNA detected more than one anti-EGFR resistance alteration in 93% of patients (9). The presence of multiple

resistance alterations complicates efforts to design a rational therapeutic strategy to overcome resistance.

Although molecular heterogeneity is a barrier to overcoming resistance, cfDNA has also identified a therapeutic opportunity. Siravegna et al. (7) found that *RAS* mutations emerge in cfDNA during anti-EGFR therapy, but they also disappear over time after discontinuing anti-EGFR therapy. Parseghian et al. (87) analyzed cfDNA from 135 patients with *RAS* wild-type metastatic CRC who progressed on EGFR antibodies and had acquired *RAS* and/or *EGFR* mutations. After withdrawal of anti-EGFR therapy, the decay half-life for an acquired *RAS* mutation was 3.4 months, while the decay half-life for an acquired *EGFR* mutation was 6.9 months.

Treatment rechallenge: Repeat treatment with a therapy after prior progression on the same therapy. Rechallenge occurs after intervening treatment(s) or a period of time has transpired

The disappearance of *RAS* or *EGFR* mutant alleles provides an opportunity for EGFR antibody treatment rechallenge. Already, clinical trials have shown potential benefit from this approach. In the single-arm phase II CRICKET trial (Cetuximab Rechallenge in Irinotecan-Pretreated mCRC, *KRAS*, *NRAS* and *BRAF* Wild-Type Treated in First Line with Anti-EGFR Therapy), 28 patients who progressed on prior anti-EGFR therapy were rechallenged with cetuximab and irinotecan (88). The response rate for cetuximab rechallenge was 21%. Notably, only those patients who had *RAS* and *BRAF* wild-type ctDNA at the time of rechallenge responded. Additionally, patients with *RAS* wild-type ctDNA prior to anti-EGFR rechallenge had significantly longer PFS than those with *RAS* mutated ctDNA (4.0 months versus 1.9 months; $p = 0.03$). Building on these results, the phase II single-arm CHRONOS trial (NCT03227926) will assess the response rate of panitumumab rechallenge in patients with *RAS* wild-type metastatic CRC who progress on first-line anti-EGFR therapy and then experience a >50% reduction in *RAS* mutational load after second-line treatment (89). Within the United States, the randomized phase II PULSE trial (Panitumumab Rechallenge Versus Standard Therapy After Progression on Anti-EGFR Therapy in Patients with Metastatic CRC; NCT03992456) will assess overall survival with panitumumab rechallenge versus standard-of-care therapy (TAS-102 or regorafenib) in patients who have no EGFR resistance-conferring alterations detected in blood. Additional anti-EGFR treatment rechallenge trials are ongoing (90).

In addition to anti-EGFR treatment rechallenge, novel therapies have been designed to overcome anti-EGFR resistance. Sym004 is a mixture of two monoclonal antibodies—futuximab and modotuximab—that bind nonoverlapping EGFR ECD epitopes (91). Despite preclinical evidence that Sym004 offers potent anti-EGFR activity, a randomized phase II trial failed to demonstrate survival benefit (92). Another therapeutic strategy to overcome *RAS* and *EGFR* ECD mutations includes the combination of a MEK inhibitor and an EGFR antibody. Already, preclinical studies have demonstrated that dual EGFR and MEK inhibition can reverse acquired anti-EGFR resistance (75, 93). A phase II clinical trial is ongoing to evaluate the response rate of panitumumab and the MEK inhibitor trametinib in patients with acquired *RAS* or *BRAF* mutations (NCT03087071). Additional studies are ongoing to target other drivers of acquired anti-EGFR resistance, including *MET* amplification (NCT03592641). Many of these therapeutic strategies leverage cfDNA assays to identify and target key resistance alterations.

CONCLUSION

Within the past decade, the clinical use of commercial blood-based cfDNA assays has increased dramatically. For patients with metastatic CRC, these assays are particularly useful when a rapid turnaround time is needed, access to tumor tissue is limited, or the patient has been exposed to anti-EGFR therapy. Due to the convenience and speed of blood-based cfDNA assays, oncologist satisfaction with cfDNA assays is high (53). As the clinical applications of cfDNA assays grow, these assays will increasingly be seen as either complementing or replacing tissue-based assays.

Despite rapid uptake of cfDNA assays in the clinic, questions remain. Given the inherent differences between tissue- and blood-based molecular assays, it is unclear if the need for tissue biopsy is obviated. Additionally, cfDNA assays perform best when ctDNA shedding is greatest. In situations where ctDNA shedding is lower, the threshold for sample adequacy is unclear. In the metastatic setting, sample adequacy is particularly vital for ruling out *RAS* and/or *BRAF* mutations. Finally, prospective studies to demonstrate the clinical utility of cfDNA to guide adjuvant treatment decision making are needed. These questions not only impact the clinical utility of cfDNA for the management of CRC but also have broader implications for the use of this technology to manage other solid tumors.

Until now, clinical use of cfDNA assays has focused on detecting DNA in blood. The ultrasensitive assays developed for DNA detection may also detect other analytes of interest, including RNA, exosomes, and cellular proteins. Additionally, recent reports have demonstrated that cfDNA assays detect tumor mutational burden (94) and microsatellite status (95) (relevant in evaluation for immunotherapy in the management of solid tumors). Finally, there are clinical scenarios where other body fluids (ascites, urine, cerebrospinal fluid, stool, etc.) may be easier to obtain or yield greater quantities of tumor-based analytes. For malignancies with low ctDNA shedding into the bloodstream—for example, malignancies confined to the central nervous system—these new assays may permit analysis of these fluids either alone or in combination with blood.

Current efforts to establish the clinical utility of cfDNA have focused on MRD detection and guiding the management of patients with advanced disease; however, several new clinical applications are possible. Already, ultrasensitive assays are being developed for enhanced cancer screening and early detection of asymptomatic malignancy (96). These minimally invasive early detection assays have the potential to transform cancer screening. As the clinical applications of cfDNA assays expand, the use of these assays may extend beyond the medical oncology clinic to primary care and other specialties. CfDNA technologies have the potential to drive future precision medicine innovations and transform medicine.

SUMMARY POINTS

1. Blood-based assays of cfDNA offer several advantages over assays of tumor tissue, including cost, convenience, and safety.
2. Postoperative ctDNA detection is a sign of poor prognosis that outperforms traditional risk factors for classifying recurrence risk.
3. Following resection of colorectal cancer, ctDNA positivity precedes biochemical or radiographic recurrence.
4. Clearance of residual ctDNA with adjuvant chemotherapy offers favorable survival benefit in initial studies; these findings need validation in a prospective clinical trial.
5. For patients with metastatic disease, quantitative changes in ctDNA predict subsequent radiographic response.
6. In patients with *RAS* wild-type metastatic CRC, cfDNA identifies key genomic drivers of anti-EGFR resistance.
7. Genomic drivers of acquired anti-EGFR resistance detected in blood will decay and eventually disappear after anti-EGFR therapy is discontinued.
8. Blood-based assays of cfDNA may identify patients who will benefit from anti-EGFR treatment rechallenge.

DISCLOSURE STATEMENT

J.H.S. serves on the consulting/advisory board of Amgen, Bayer, Genentech/Roche, Seattle Genetics, *Proteus* Digital Health, Chengdu Kanghong Biotechnology, and Natera. He receives research funding from Seattle Genetics, AstraZeneca, Genentech/Roche, OncoMed, Abbvie, Exelixis, Macrogenics, Leap Therapeutics, MedImmune, Amgen, and Nektar. V.K.M. serves on the consulting/advisory board of Array Biopharma and Pfizer. He receives research funding from Array Biopharma, Bristol Myers-Squibb, EMD Serono, AstraZeneca, and Immatics.

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