Current Perspectives on Circulating Tumor DNA, Precision Medicine, and Personalized Clinical Management of Cancer

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ABSTRACT

Circulating tumor DNA (ctDNA) has recently emerged as a minimally invasive “liquid biopsy” tool in precision medicine. ctDNA-genomic DNA fragments that are released into the bloodstream after the active secretion of microvesicles or tumor cell lysis reflect tumor evolution and the genomic alterations present in primary and/or metastatic tumors. Notably, ctDNA analysis might allow the stratification of patients, the monitoring of the therapeutic response, and the establishment of an opportunity for early intervention independent of detection by imaging modalities or clinical symptoms. As oncology moves towards precision medicine, the information in ctDNA provides a means for the individual management of the patient based on their tumor’s genetic profile. This review presents current evidence on the potential role for ctDNA in helping to guide individualized clinical treatment decisions for patients with melanoma, castration-resistant prostate cancer, breast cancer, metastatic colorectal cancer, and non-small cell lung cancer.

Background

Advances in molecular biology have increased the efficiency of the clinical management of patients with cancer. The molecular characterization of tumors is a key signature for the clinical application of individualized treatment strategies. In this context, the emergence of precision medicine allows geneticists and oncologists to select the personalized/individual treatments based on the genetic profile (1).

Even though tissue biopsy remains the gold standard for the molecular stratification of tumors, this procedure is often difficult to perform, particularly in relapsed and metastatic settings, and does not support intratumoral heterogeneity. Moreover, driver mutations might exhibit changes during tumor development or treatment, and thus, clonal evolution needs to be evaluated through tumor re-biopsy monitoring (2). Figure 1 shows intratumoral heterogeneity and clonal evolution.

Liquid biopsy has emerged as a promising minimally invasive tool for precision medicine due to its ability to provide multiple global snapshots of primary and metastatic tumors at different times and more representative images of the spatial and temporal tumor heterogeneity (3, 4).

Accumulating evidences have demonstrated potential applications of circulating tumor DNA (ctDNA) analysis obtained from plasma at multiple time points throughout the natural course of cancer development, diagnosis, and treatment. ctDNA refers to cell-free DNA (cfDNA) fragments that are released into the circulation from primary tumor or metastatic cells; these fragments have a length of approximately 166, 332, or 498 base pairs and exhibit tumor-specific alterations, including point mutations, chromosomal rearrangement, copy-number variation (CNV), and DNA methylation (3, 5). These molecules are released into the bloodstream as a result of the active secretion of cell microvesicles, the apoptosis or necrosis tumor cells, and the systemic treatment. A ctDNA analysis might allow the stratification of patients according to the risk of tumor relapse as well as early intervention independent of detection by imaging exams or clinical symptoms. As oncology moves towards precision medicine, the information in ctDNA provides a means for the individual management of the patient based on their tumor’s genetic profile.

Figure 2 illustrates the landmark-discoveries about ctDNA and its use in precision medicine (6, 8–16).

Interestingly, ctDNA has a short half-life in the bloodstream, and this characteristic makes it a very important tool in analyzing real-time situation of mutation dynamics and tumor burden after surgery or systemic treatment. A ctDNA analysis might allow the stratification of patients according to the risk of tumor relapse as well as early intervention independent of detection by imaging exams or clinical symptoms. Thus, real-time tumor dynamics might be monitored through ctDNA analysis for early prediction and assessment of drug response in patients with cancer (3).

In addition, ctDNA also have been reported as a screening tool able to predict carcinogenesis. For instead, Scimia and colleagues (17) reported Colo Scape Kit (DiaCarta) to detect mutations in the APC, KRAS, BRAF, and CTNNBI genes in plasma as a promising tool to...
detect cancers and advanced adenomas in a triage of fecal immunochemical test positive (FIT+) patients.

Numerous methods classified into two broad groups based on the scale of analysis next-generation sequencing (NGS) and single or multiplexed locus assays can be used to identify alterations in ctDNA with different sensitivity levels (Fig. 3). NGS-based methods may detect novel genetic aberrations or multiple mutations simultaneously. In contrast, single or multiplexed locus assays identify only one or a few genetic mutations. PCR-based techniques widely used due to reasonable cost, particularly digital droplet PCR (ddPCR) have higher sensitivity and specificity than NGS (3, 18).

In this review, we provide an overview of the status of the field of ctDNA and its role in the selection and the management of the treatment for melanoma, prostate cancer, breast cancer, colorectal cancer, and lung cancer. Furthermore, we discuss the advantages and limiting factors associated with the application of ctDNA as precision medicine tool in clinical practice.

**Biomarkers for the Clinical Management of Metastatic Melanoma**

In the last years, chemotherapy for malignant melanoma has been superseded or supplemented by systemic immunotherapies, such as anti-PD-1 drugs (nivolumab and pembrolizumab) and anti-CTLA-4 antibody (ipilimumab), and targeted therapies, such as BRAF ( vemurafenib, dabrafenib, and encorafenib) and/or MEK inhibitors (cobimetinib, trametinib, and binimetinib). However, targeted therapies
Timeline of ctDNA research advancement. Nucleic acids were first identified in the human circulation in 1948 (8). Decades later, the first association between high concentrations of ctDNA and the presence of cancer was described by Leon and colleagues (9). In 1989, Stoun and colleagues (10) found neoplastic alterations in ctDNA from patients with NSCLC. Recently, Lupini and colleagues (16) developed an enhanced-ice-COLD-PCR (E-ice-COLD-PCR)-based method to improve the sensitivity of ctDNA detection. In 2019, Therascreen PIK3CA RQG PCR Kit receives U.S. regulatory approval for its use in guiding treatment decisions from tissue and liquid biopsy for alpelisib newly approved therapy for breast cancer.

In clinical practice, the identification of BRAF mutations, mainly valine-to-glutamic acid substitution (V600E) or valine-to-lysine substitution (V600K) at codon 600, is required for the administration of BRAF and MEK inhibitors through the sequencing of melanoma tissue (20).

Previous studies have demonstrated that the presence of BRAF mutation in ctDNA can be quite useful for the real-time monitoring of the tumor genome and provides information regarding the targeted therapy of malignant melanoma to immunotherapy and targeted therapy that complements the usual tissue biopsy results (21, 22). Moreover, it has been reported that the incorporation of ctDNA in the clinical management of melanoma provides early and specific information about tumor growth, and the combination of ctDNA information with radiographic imaging exams could increase the utility of current precision medicine (21).

Garlan and colleagues (23) studied metastatic melanoma BRAFmut patients (a subgroup of VEUUMELA) treated with vemurafenib, which is a first-line therapy for patients with metastatic melanoma BRAF^{V600E}. Interestingly, ctDNA concentrations in plasma were inversely correlated to the drug concentrations that are given to the patient during treatment. Thus, these researchers proposed that monitoring of ctDNA concentrations in plasma during follow-up could serve as a novel approach to tumor evolution in patients with melanoma treated with anti-BRAF therapies. In addition, NRAS mutations have been detected in 28% of metastatic melanoma patients, and specific mutations in codon 61 (Q61K, Q61R, Q61L, Q61H) have been associated with resistance to several drugs (24). A preclinical study showed that melanoma cell lines with both BRAF^{V600E} and NRAS^{Q61K} mutations are resistant to vemurafenib but sensitive to selumetinib (25). Moreover, NRAS^{Q61K/R} mutations are evident in the ctDNA of patients who develop resistance to BRAF inhibitor treatments (20, 26).

Recently, Keller and colleagues (27) detected tumor progression or regression in patients with melanoma stage I/II to IV treated with immunotherapy (anti-CTLA-4 and/or anti-PD1) by ctDNA monitoring using ddPCR technique to quantify BRAF (V600E and V600K) and NRAS (Q61K, Q61R, Q61L, and Q61H) mutations. In 2017, Ashida and colleagues (28) observed that one patient with multiple metastases had a BRAF^{V600E} mutation in plasma ctDNA, and after 21 days of immunotherapy with nivolumab, the ctDNA levels declined to undetectable levels.

In addition to the clinical monitoring of the therapy response of patients with metastatic melanoma by early detection of ctDNA variations during immunotherapy, the increased ctDNA levels might benefit poor progression free survival (PFS), and overall survival (OS). Lee and colleagues (29) detected ctDNA in 53% (40/76) of patients with metastatic melanoma at baseline and reported ctDNA as a predictor of tumor response, PFS and OS, receiving treatment with PD1 inhibitors alone or in combination with ipilimumab. Elevated ctDNA detected during treatment pointed to patients experiencing a poor prognosis. These findings suggest that ctDNA application in immunotherapy context directs professionals for a better decision-making treatment in advanced melanoma clinical setting. Accordingly, these studies demonstrate that mutation monitoring in ctDNA from patients with melanoma could detect a higher tumor burden and help oncologists in personalize therapy lines even before disease progression is observed by imaging exams.

### AR Gene in Patients with Metastatic Castration-Resistant Prostate Cancer

Androgen hormones regulate the growth of normal and malignant prostate tissue via activating androgen receptor (AR) signaling in epithelial and stromal cells. This steroid hormone receptor plays an essential role in the development, progression and therapeutic response of prostate cancer (30). First, all patients with metastatic prostate cancer are submitted to an androgen deprivation therapy (ADT) using gonadotropin-releasing hormone agonist (GnRH) or bilateral orchectomy. However, this treatment effect is transient and eventually result in progression to metastatic castration-resistant prostate cancer (mCRPC), which is directly linked to increased levels of AR amplification or mutations that confer resistance to androgen.
deprivation therapy (31). mCRPC is a lethal clinical state and these patients to receive therapy with first-generation (flutamide, nilutamide, and bicalutamide) or second-generation (enzalutamide and apalutamide) AR antagonists or with abiraterone, an androgen biosynthesis inhibitor. The treatment landscape of mCRPC is complicated and confounded by partial and unpredictable cross-resistance between AR-targeted therapy and a shifting consensus regarding when taxane-based chemotherapies should be used (32, 33). Molecular subtyping breakthroughs have spurred clinical trials of targeted agents aimed at distinct genotypes and demonstrate the need for real-time and practical tumor biomarkers to guide therapy selection (34).

Several studies have observed consistent mutations and CNV in ctDNA of patients with mCRPC, including in the AR gene (31, 35–39). Four AR point mutations (L702H, H874Y, T877A, and T878A) found in ctDNA have been associated with resistance to abiraterone (35, 38). These AR gene aberrations suggest that ctDNA can help identify representative of tumor clones responsible for driving disease progression in CRPC. In addition, AR F877L mutation in ctDNA of patients with mCRPC has been reported as a resistance mechanism to enzalutamide treatment (35, 40). According to Azad and colleagues (35), who investigated AR amplification in ctDNA through array comparative genomic hybridization, AR amplification is significantly more common in ctDNA of patients with mCRPC progressing on enzalutamide (53%) than on abiraterone (17%) or other agents (21%).

In 2017, Wyatt and colleagues (39) performed systematic comparisons between ctDNA and respective solid biopsies from 45 patients with mCRPC and suggested that ctDNA assays could be confidently used for molecular stratification and establishing a better clinical management program for patients. In their study, the authors performed targeted sequencing of 72 driver genes clinically relevant for mCRPC and found that 65% (29/45) of patients exhibited AR amplifications. These researchers also revealed that all somatic mutations found in matched metastatic tissue biopsies were present in plasma ctDNA. This robust concordance suggests that ctDNA assays can be reliably used as a tool for the stratification of patients for clinical purposes.

Using ddPCR assays for the AR copy number and mutations on sequential plasma DNA samples from two independent cohorts, Conteduca and colleagues (36) evaluated AR alterations in ctDNA of patients with CRPC. In the primary cohort, AR gain was observed in 14% (10/73) of chemotherapy-naïve and 34% (33/98) of patients with post-docetaxel CRPC treated with enzalutamide or abiraterone. This gain was associated with worse OS and PFS, and a decreased likelihood of a ≥50% decline in PSA. AR L702H and AR T878A have been reported in 11% (8/73) of patients after post-docetaxel treatment. Similar to the results for the prechemotherapy group of the primary cohort, the analysis of the secondary cohort, an arm of PREMIERE trial (NCT02288936), found AR amplification in 12% (11/94) of patients, and this amplification was significantly associated with shorter
Early and Minimally Invasive Prediction of the Treatment Response to Targeted Therapy and Immunotherapy in Non–Small Cell Lung Cancer

For metastatic non–small cell lung cancer (mNSCLC), the development of targeted therapies has changed the treatment paradigm. The National Comprehensive Cancer Network (NCCN) guidelines (v03.2019) recommend nine biomarkers for patients with mNSCLC: EGFR activating mutations, ALK fusions, ROSI fusions, BRAFV600E mutation, RET fusions, MET amplification, MET exon 14 skipping variants, HER2 mutations, and NTRK rearrangements (available at NCCN.org). Around 10% of NSCLC harbor EGFR mutations and up to 90% of these mutations are located in exon 19 as deletions (present as deletions) and exon 21 (L858R). These alterations are sensitive to systemic EGFR tyrosine kinase inhibitors (EGFR-TKI) treatment with gefitinib or erlotinib at the first-generation agent. Second-generation agents, such as afatinib, are recommended for patients with advanced NSCLC with EGFRL858R in tumor tissue (44). Furthermore, the other 10% of patients have uncommon EGFR mutations (G719X, E709X, L861Q, RAD51, A763_Y764insFQEA, S768I, E709X indel, exon19 insertion, KDD duplication, RAD51 rearrangement, and insertion in exon 20) and the EGFR-TKI therapy response varies significantly according to the mutation (45).

Unfortunately, resistance to therapy is a common occurrence of anticancer treatment and limits the long-lasting use of targeted therapies. Thus, even with the impressive high response rates to first-line EGFR-TKI, disease progression appears within 9 to 13 months (46). Therefore, second-line therapies focusing on resistance mutations are often required, but tissue profiling is needed to identify specific acquired genomic alterations. Approximately 60% of these cases, the acquired resistance is due to a second point mutation at position 790 involving the substitution of threonine to methionine (T790M) in EGFR (47).

For patients with NSCLC showing progression and EGFR T790M, the FDA approved osimertinib, an oral, irreversible third-generation EGFR-TKI in 2015. Recently, osimertinib showed superiority as a first-line therapy to improve the PFS of EGFR mutation-positive patients compared with first-generation EGFR-TKIs (18.9 months vs. 10.2 months, respectively; ref. 48). These data note to a drastic change in the treatment decision based on whether osimertinib should be considered a first-line treatment for NSCLC EGFR-mutated patients. Accordingly, EGFR C797S is the major mutation associated with acquired resistance to the first-line agent osimertinib, and in the future, a ctDNA analysis should focus on EGFR C797S activating mutation to determine the treatment (49).

In June 2016, the FDA approved cobas EGFR Mutation Test v2 (P150047 – Roche Molecular Systems), a RT-PCR assay that can identify 42 different EGFR gene mutations present in exons 18 to 21 with detection limits that differ between tissue-derived DNA and plasma-derived ctDNA samples from patients with NSCLC (ENSURE study; ref. 50). ctDNA has been recommended as a good alternative to conventional tissue biopsy analysis for the identification of EGFR mutations and for guiding treatment with specific EGFR-TKIs as the first-line treatment. Figure 4 summarizes the dynamic of EGFR mutation detection in ctDNA of patients with NSCLC.

Recently, Liquid Lung-O-Cohort 2 trial evaluated the efficacy of osimertinib through ctDNA analysis from patients with NSCLC using the cobas EGFR Mutation Test v2 (P150044 – Roche Molecular Systems) and recommends plasma ctDNA as a real alternative to tumor biopsy in current clinical practices for the screening of acquired EGFR T790M resistance mutation (51). Notably, patients with NSCLC whose tumors harbor rearrangements in ALK or ROSI also benefit from targeted therapies. ALK and ROSI kinases are phylogenetically similar, and both are sensitive to first-generation ALK inhibitor therapy, namely crizotinib, as the first- and second-line treatment (52). Nearly 7% of all NSCLC cases show rearrangements in ALK, whereas ROSI rearrangement is detected in approximately 2% of patients with NSCLC (53). Despite enormous efforts, patients eventually develop resistance after 9 to 10 months of ALK-inhibitor treatment through several mechanisms, such as ALK mutations (II171N, L1196M, G1269A, and F1174L), amplification of the rearranged fusion gene EML4-ALK, or the activation of bypassing signal pathways. After the acquisition of resistance to crizotinib, second-generation ALK inhibitors (ceritinib and alectinib) could be used. In 2017, FDA-approved alectinib was routinely used as the first-line therapy for metastatic ALK-positive NSCLC based on results from the Phase III ALEX study (54).

In particular, the ROSI fusion was paired to 14 different partner genes in NSCLC (CD74, SLC34A2, SDC4, EZR, FIG, TPM3, LRG3, KDEL2, CCDC6, MSN, TMEM16B, TPDS2L1, CLTC, and LIMA1), but CD74-ROSI occurs most frequently in lung cancer. Nevertheless, ROSI C2032R is the most common resistance mutation observed after crizotinib treatment (55).

Regarding ROSI rearrangement, Dagogo-Jack and colleagues (56) found 100% concordance in detection of ROSI–CD74 fusion between tumor tissue and plasma ctDNA, and 50% sensitivity for the detection of this fusion after relapse. In addition, Niou and colleagues (57) detected CD74–ROSI fusion at baseline in ctDNA from patients with NSCLC treated with crizotinib. In 2017, Wang and colleagues (58) detected CD74–ROSI fusion in ctDNA, whereas cytotoxic chemother-apy typically the decreased ctDNA levels. Taken together, these data suggest that ctDNA is a promising approach for the detection of ROSI fusions. To date, few data are available for ALK mutations in plasma ctDNA from patients with NSCLC. The replacement of isocitrate by asparagine at amino acid residue 1171 (II171N) was found in a patient who showed disease progression after to alectinib therapy and then responded to ceritinib. Moreover, one patient who showed progression...
after crizotinib treatment exhibited resistant mutations ALK1152R, ALKI1171T, and ALKL1196M (59, 60). In this context, the ctDNA analysis performed by ddPCR was shown to be a low-cost procedure that can perform more sensitive detection from a peripheral blood sample and avoids re-biopsy of the tissue tumor due to the inadequacy of its local access. Nevertheless, ROS1G2032R has been described as the most common resistance mutation that emerges post-crizotinib (55, 57).

Other gene mutations detected in ctDNA isolated from plasma have been identified in approximately 1% to 2% of NSCLC cases, including AKT, DDR2, FGFR, HER2, MEK1, MET, NTRK1, PI3KCA, PTEN, and RET. According to the LOXO-TRK-1400, SCOUT, and NAVIGATE studies, the FDA approved the use of larotrectinib for patients with fusion of the NTRK gene and no resistance mutations (61). Helman and colleagues (62) described one patient had stable disease for 168 days while receiving rociletinib treatment but experienced systemic progression on day 229 due to an increase in target lesions, as demonstrated by imaging. Six days prior to progression, the TPM3–NTRK1 fusion emerged in ctDNA at 0.14% VAF and continued to increase.

The inclusion of a ctDNA analysis in the management of NSCLC provides strong evidence of the early treatment response, and thereby provides a basis for determining disease progression and the need for changes in treatment. Numerous recent studies have verified the benefit of Guardant360 for planning the therapeutic protocol for patients with NSCLC. Guardant360 is the first clinically validated liquid biopsy assay that allows a comprehensive molecular analysis based on the evaluation of ctDNA including eight genetic markers recommended by NCCN (EGFR, ALK, ROSI, BRAF, RET, MET, HER2, and NTRK) that are useful for guiding doctors to the appropriate therapies. In the NILE study, Guardant360 demonstrated more than 90% concordance for four biomarkers between FDA-approved therapies and standard-of-care tissue biopsy, EGFR mutations (83%), ALK fusions (80%), ROSI fusions (58%), and BRAFV600E (35%), in a cohort of 282 patients with mNSCLC at 28 centres in the United States. This prospective multicentre study evidenced that the use of Guardant360 for patients with newly diagnosed mNSCLC successfully identifies the recommended biomarkers at a rate as high as that obtained with standard-of-care tissue testing and provides these results significantly faster and for a significantly higher proportion of the population (63). In addition to NSCLC, this ctDNA test aims to assist the monitoring of the drug response and therapeutic management in different solid tumors (64, 65).

It is important to highlight that the tumor mutational burden on plasma (bTMB; ctDNA-based) could serve as a potential biomarker of immune checkpoint inhibitors efficacy in patients with NSCLC. According to Gandara and colleagues (66), bTMB has accurately and reproducibly clinical application as a predictive biomarker for PFS. In 2019, Wang and colleagues (67) reported bTMB ≥ 6 was associated with superior PFS and objective response rates. Furthermore, Anagnostou and colleagues (68) observed that early ctDNA clearance was a significant prognostic factor for PFS and OS, analyzing serial blood samples from 38 patients with NSCLC during immune checkpoint blockade or after a clinical trial of neoadjuvant nivolumab. Indeed, the authors predicted clinical outcomes on 8.7 weeks approximately before computed tomography imaging status. Since ctDNA dynamics could determine the pathologic response in immune checkpoint inhibitor setting, these findings reaffirm the ctDNA approach as a practical tool in immunotherapy treatment.
**ESR1 and PIK3CA Mutations as Resistance Biomarkers for Endocrine Therapy in Hormone Receptor-Positive Patients with Breast Cancer**

Overall, approximately 80% of breast tumors express hormone receptor (HR), estrogen receptor (ER), or progesterone receptor (PR), at time of diagnosis and are treated with endocrine therapy, including tamoxifen, aromatase inhibitors, and fulvestrant (69). However, approximately one-third of ER-positive (ER+) patients show poor response to these therapies due to innate or acquired drug resistance. These critical resistance mechanisms are associated with activating mutations in the ESR1 gene, mainly in codons 536 to 538 (70). Mutations in ESR1 have been reported to promote ER transcriptional activity in an estrogen-independent manner. ESR1 mutations are present not only in breast cancer but also in metastatic breast cancer (MBC) (71, 72). These alterations could be selected from rare mutant clones during treatment and induce resistance, which favor metastasis. Analysis of these alterations in ctDNA is a potential approach for detecting these mutations and thus selecting a suitable treatment (16).

In 2016, BOLERO-2 double-blind phase III trial determined the prevalence of ESR1 mutations in ER+ patients with metastatic breast cancer (MBC) treated with exemestane together with everolimus. ESR1 mutations was the most common mutation found in about 22% (114/541) of patients. However, the addition of mTOR inhibitor showed failure in patients with breast cancer with ESR1 mutations. These results demonstrated that ESR1 mutations are prevalent in ER+ aromatase inhibitor-treated MBC, in which only one or both mutations are associated with a reduced median OS and more aggressive disease. Therefore, the ESR1 mutation status might represent a potential biomarker for prediction of drug–response evolution in cancer therapy as well as for prognosis of ER+ patients (73).

To improve the sensitivity of detection in ctDNA, Lupini and colleagues (16) developed an enhanced-ice-COLD-PCR (E-ice-COLD-PCR)-based method for the enrichment of ESR1 gene mutations. Initially, this method demonstrated potential for increasing the frequency of ESR1 mutant alleles (Y537S, D538G, and Y537C) in metastatic samples. An analysis of ctDNA from 56 patients with ER+/MBC was performed in the hotspot region of the ESR1 gene by E-ice-COLD-PCR amplification. Then, resulting amplicons were analyzed using both NGS for all mutations and ddPCR for the Y537S variant. This approach revealed that 27% (15/56) of the ctDNA samples harbored a mutation in codons 536 to 538 (L536H, Y537S, D538G, Y537N, Y537H, and L536Q), confirming the efficiency of the combination of E-ice-COLD-PCR and NGS to enrich and detect all possible alterations present in ESR1 codons 536 to 538 without any prior knowledge. To evaluate ESR1Y537S variant, both NGS and ddPCR showed consistent results. Moreover, ESR1 mutations were found only in 13% (6/46) of tissue biopsies, which indicated that heterogeneity is more faithfully represented in ctDNA than in a tissue biopsy. Taken together, these results suggest that E-ice-COLD-PCR coupled with NGS could serve as a possible method for the real-time monitoring of patients with metastatic and nonmetastatic ER+ breast cancer in clinical practice. This approach could allow the early detection of tumor clones that develop resistance to endocrine therapy and might aid accurate therapy adjustments.

Additional to ESR1 mutations, PIK3CA mutations in plasma ctDNA also has been reported as a minimally invasive method to quickly assess and monitor endocrine therapy resistance in patients with MBC (74). Moreover, PIK3CA mutations have been suggested as predictive biomarkers for PI3Kα-selective inhibitor treatment (75). More than 80% of all PIK3CA mutations are located within exon 9 (E545K and E542K) and exon 20 (H1047R and H1047L) (76). In 2017, García-Saenz (77) found a good agreement between plasma and FFPE samples when assessing PIK3CA mutation status in advanced patients, using array-based digital PCR. Recently, O’Leary and colleagues (78) observed good agreement in ESR1e PIK3CA mutation status in plasma samples from patients with advanced breast cancer enrolled in the phase III PALOMA-3 trial between BEAMing and ddPCR, suggesting sufficient reproducibility for clinical use.

**Figure 5.** Clinical use of ctDNA to guide novel treatment line in HR+, HER2−, PIK3CA-mutated advanced breast cancer. If HR+, HER2−, advanced breast cancer patients with progression on or after an endocrine-based regimen harbor PIK3CA-mutation in the tumor must be treated with alpelisib in combination with endocrine therapy fulvestrant. Tissue biopsy or ctDNA PIK3CA status is determined by therascreen PIK3CA RQq PCR Kit. Moreover, ctDNA analysis could allow real-time monitoring of PIK3CA mutation before disease progression.
On May 24, 2019, the FDA approved the combination of alpelisib with the endocrine therapy fulvestrant to treat postmenopausal women and men, with HR+, HER2−, PIK3CA-mutated, advanced or MBC following progression on or after an endocrine-based regimen. Concomitantly, FDA also approved the therascreen PIK3CA-RQ PCR Kit (P190004; Qiagen), a qualitative PCR assay for the detection of 11 mutations in the PIK3CA gene in ctDNA and/or tumor tissue. Patients who are negative by the therascreen PIK3CA RQ PCR Kit using the liquid biopsy should undergo tumor biopsy (Fig. 5; ref. 75).

### The Use of Anti-EGFR Antibody Is Guided by all RAS Mutations in Metastatic Colorectal Cancer

Colorectal cancer is the third most common solid malignancy worldwide, and its treatment options are dependent on the colorectal cancer stage, performance status, and molecular tumor landscape. In metastatic colorectal cancer, clinical use of anti-EGFR mAbs exhibits increased treatment efficiency. However, anti-EGFR therapies have shown efficiency in KRAS wild-type tumors cells subpopulations (79).

Almost half of all colorectal cancer have KRAS mutations, and the current standard of care is to determine the KRAS mutation status in tumors before any therapy decision because this status is critical for the detection of innate resistance to treatment (80). It has been established that the KRAS mutations located in codons 12 (G12V, G12A, G12D, G12S, G12C, and G12R) and 13 (G13D) correspond to 90% of all KRAS mutations in colorectal cancer. Moreover, these mutations are responsible for drug resistance, particularly cetuximab and panitumumab, which are monoclonal antibodies directed against EGFR and other standard components in chemotherapy protocols (81). During treatment, several patients develop resistance to anti-EGFR therapies. For patients with disease progression harboring RAS mutations, second-line therapy is recommended, that is, bevacizumab or drugs with broader activity such as aflibercept, regorafenib, and VEGF inhibitors (82).

In 2015, the FDA approved cobas KRAS Mutation Test (P140023; Roche Molecular Systems) as a companion diagnostic tool for identification of the correct molecular information from metastatic colorectal cancer tissue. The test aims to detect mutations in codon 12, 13, and 61 of KRAS gene and can be performed in approximately 8 hours, and thus enabling physicians to make informed and rapid decisions regarding the treatment choice. However, the present method still shows some limitations with respect to clinical monitoring and the assessment of spatial and temporal heterogeneity.

Numerous studies have recommended the routine detection of KRAS mutations in ctDNA from patients with colorectal cancer as an alternative method to monitoring, predicting the response of the EGFR blockade, or prognostic prediction (81, 83–85). In 2016, Zill and colleagues provided a review on the use of liquid biopsy in metastatic colorectal cancer, emphasizing the use of KRAS, NRAS, PIK3CA, and BRAF analysis for determining treatment options (86).

### Table 1. ctDNA clinical approach in metastatic melanoma, mCRC, mNSCLC, HR+ breast cancer, and metastatic colorectal cancer.

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<thead>
<tr>
<th>Cancer</th>
<th>ctDNA</th>
<th>Clinical implication</th>
<th>Reference</th>
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<tr>
<td>Metastatic melanoma</td>
<td><strong>BRAF</strong>&lt;sup&gt;V600E&lt;/sup&gt;, <strong>V600K</strong></td>
<td>Clinical monitoring of immunotherapy and targeted therapy</td>
<td>(20, 21, 23, 25–28, 34)</td>
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<td><strong>NRAS</strong>&lt;sup&gt;G61K&lt;/sup&gt;, **Q61R&lt;/sup&gt;, **Q61L&lt;/sup&gt;, **Q61H&lt;/sup&gt;</td>
<td>Early biomarker of tumor evolution</td>
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<td>mCRC</td>
<td><strong>AR</strong>&lt;sup&gt;A102Q&lt;/sup&gt;, **H874Y&lt;/sup&gt;, **T877A&lt;/sup&gt;, **T878A&lt;/sup&gt;</td>
<td>Resistance mechanism to abiraterone treatment</td>
<td>(35, 38)</td>
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<td></td>
<td><strong>AR</strong>&lt;sup&gt;A879L&lt;/sup&gt;</td>
<td>Resistance mechanism to enzalutamide treatment</td>
<td>(35, 40)</td>
</tr>
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<td></td>
<td><strong>AR</strong>&lt;sup&gt;amplification&lt;/sup&gt;</td>
<td>Association with decreased OS and PFS before starting enzalutamide or abiraterone</td>
<td>(36, 39, 42)</td>
</tr>
<tr>
<td>mNSCLC</td>
<td><strong>EGFR</strong>&lt;sup&gt;T790M&lt;/sup&gt;</td>
<td>Resistance mechanism to first-line EGFR-TKI, and sensibility to osimertinib</td>
<td>(47, 48)</td>
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<td></td>
<td><strong>EGFR</strong>&lt;sup&gt;C797S&lt;/sup&gt;</td>
<td>Acquired resistance to osimertinib</td>
<td>(49)</td>
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<td></td>
<td><strong>CD74-ROS1 fusion</strong></td>
<td>Resistance mechanism to crizotinib treatment</td>
<td>(59, 60)</td>
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<td></td>
<td><strong>ALK</strong>&lt;sup&gt;107N&lt;/sup&gt;</td>
<td>Resistance mechanism to alecetinib treatment and response to ceritinib</td>
<td>(62)</td>
</tr>
<tr>
<td>HR+ breast cancer</td>
<td><strong>ROS1</strong>&lt;sup&gt;E2032R&lt;/sup&gt;</td>
<td>Resistance mechanism post-crizotinib</td>
<td>(57, 59, 66, 67)</td>
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<td><strong>ESR1</strong>&lt;sup&gt;Y538G&lt;/sup&gt;</td>
<td>Resistance mechanism to exemestane plus everolimus</td>
<td>(74, 79)</td>
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<td><strong>ESR1</strong>&lt;sup&gt;Y537S&lt;/sup&gt;</td>
<td>Resistance mechanism to everolimus</td>
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<td><strong>ESR1</strong>&lt;sup&gt;Y537S&lt;/sup&gt;, **Y537C&lt;/sup&gt;, **Y537N&lt;/sup&gt;, <strong>D538G</strong></td>
<td>Association with poor prognosis</td>
<td>(16, 74)</td>
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<td><strong>ESR1</strong>&lt;sup&gt;Y537C&lt;/sup&gt;</td>
<td>Resistance mechanism to endocrine therapy</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td><strong>ESR1</strong>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>Modest effect in inducing resistance to fulvestrant</td>
<td>(74, 79)</td>
</tr>
<tr>
<td></td>
<td><strong>PIK3CA</strong>&lt;sup&gt;E545K&lt;/sup&gt;, **E542K&lt;/sup&gt;, **H1047R&lt;/sup&gt;, **H1047L&lt;/sup&gt;</td>
<td>Relative sensitivity to fulvestrant</td>
<td>(78, 79)</td>
</tr>
<tr>
<td>Metastatic colorectal cancer</td>
<td><strong>PIK3CA</strong>&lt;sup&gt;Y1042&lt;/sup&gt;</td>
<td>Predictive biomarker for PI3K-selective inhibitor treatment</td>
<td>(78, 79)</td>
</tr>
<tr>
<td></td>
<td><strong>NRAS/KRAS</strong>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>Early biomarker of treatment response</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td><strong>PIK3CA</strong>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>Predictive biomarker for specific PIK3CA inhibitor</td>
<td>(82, 84–86)</td>
</tr>
<tr>
<td></td>
<td><strong>KRAS</strong>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>Predictive biomarker for EGFR blockade</td>
<td></td>
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<tr>
<td></td>
<td><strong>NRAS point mutation</strong></td>
<td>Clinical monitoring of response to EGFR blockade</td>
<td></td>
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<tr>
<td></td>
<td><strong>BRAF</strong>&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Clinical monitoring of response to EGFR blockade</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>KRAS</strong>&lt;sup&gt;mut&lt;/sup&gt; and <strong>BRAF</strong>&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Monitoring disease and drug resistance during disease progression</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Resistance to an EGFR inhibitor</td>
<td>(87–89)</td>
</tr>
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colleagues (85) analyzed almost 15,000 ctDNA samples from patients with colorectal cancer and found that KRAS<sup>G12</sup>, KRAS<sup>G13</sup>, and KRAS<sup>261</sup> were the most common resistance alterations. In particular KRAS<sup>G12V</sup>, one of the most frequent mutation subtypes, has been studied as an alternative biomarker in plasma ctDNA for monitoring disease and drug resistance during disease progression. Interestingly, patients with metastatic colorectal cancer exhibit a significantly higher frequency of the KRAS<sup>G12V</sup> than nonmetastatic patients, and this variant has been associated with a worse PFS compared with wild-type KRAS (81).

Recently, Knebel and colleagues (83) described the monitoring of a patient with KRAS wild-type metastatic colorectal cancer treated with anti-EGFR therapy in combination with chemotherapy using a commercial assay designed for the detection of frequent KRAS mutations (KRAS Screening Kit; BioRad; G12V, G12D, G12A, G12C, G12R, G12S, and G13D). These researchers found KRAS mutations in ctDNA prior to clinical progression of the disease and after the first exposure to anti-EGFR therapy. Increase in the levels of KRAS mutations accompanied the evolution of the disease. Furthermore, high levels of mutations in the KRAS gene were visualized in patient who were re-challenged with FOLFOX or the fourth line of treatment and showed little or no response. These results support the hypothesis that the longitudinal monitoring of the KRAS status in ctDNA during anti-EGFR therapy can be efficiently used for the early detection of drug resistance and for guiding clinical management in the era of precision medicine.

In addition, resistance to an EGFR inhibitor can also be a consequence of colorectal cancer harboring mutations in NRAS or BRAF<sup>V600E</sup> (86–88). Activating mutations in NRAS and BRAF<sup>V600E</sup> are found in approximately 5% and 4% to 7% of colorectal tumors, respectively (89). In 2014, Thierry and colleagues (88) reported the first clinical validation of a ctDNA analysis based on detection of KRAS exon 2 (G12V, G12A, G12D, G12S, G12C, G12R, and G13D) and BRAF<sup>V600E</sup> mutations in patients with metastatic colorectal cancer using a qPCR-based method. Posteriorly, this group of researchers performed a clinical study with 140 patients with metastatic colorectal cancer and revealed that the frequencies of KRAS, NRAS, and BRAF mutants was 59%, 11.8%, and 14.4% in ctDNA analysis and 44%, 8.8%, and 7.2% in tumor-tissue analysis, respectively. These findings indicate that ctDNA analysis could be used instead of tumor-tissue analysis and that ctDNA can be utilized to considerably reduce the turnaround time for data required for clinical decisions (87).

Future Perspectives
c

cDNA analysis offers a minimally invasive approach for detection of clinically relevant genomic alterations useful to determine accurate therapies and for the real-time monitoring of treatment response in patients with different types of cancer (Table 1). To date, FDA approved ctDNA assays are available for metastatic and locally advanced NSCLC and HR+ HER2<sup>+</sup> advanced breast cancer in progression on or after endocrine therapy.

cDNA remains as a potential research tool for other cancer types.

cDNA carried out at diagnosis guides treatment to target actionable mutations and detected resistant mutation.

cDNA levels can correlate to response to therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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References

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Current Perspectives on Circulating Tumor DNA, Precision Medicine, and Personalized Clinical Management of Cancer


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