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Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet?

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Summary

The optimal choice of cancer therapy depends upon analysis of the tumor genome for druggable molecular alterations. The spatial and temporal intratumor heterogeneity of cancers creates substantial challenges, as molecular profile depends on time and site of tumor tissue collection. To capture the entire molecular profile, multiple biopsies from primary and metastatic sites at different time points would be required, which is not feasible for ethical or economic reasons. Molecular analysis of circulating cell-free DNA offers a novel, minimally invasive method that can be performed at multiple time-points and plausibly better represents the prevailing molecular profile of the cancer. Molecular analysis of this cell-free DNA offers multiple clinically useful applications, such as identification of molecular targets for cancer therapy, monitoring of tumor molecular profile in real time, detection of emerging molecular aberrations associated with resistance to particular therapy, determination of cancer prognosis and diagnosis of cancer recurrence or progression.

Keywords

liquid biopsy; cell-free DNA; advanced cancer; targeted therapy; personalized medicine

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Introduction

Despite significant progress in modern oncology, efficacy of treatment for advanced cancer remains poor; the majority of advanced tumors become resistant to available therapies and the patient ultimately succumbs to advancing metastatic disease [1]. This is mainly due to clonal evolution of the disseminated tumor and acquired resistance to cancer therapies, even if fitted to the known molecular profile [2, 3]. In the current era of personalized medicine, the optimal choice of therapy depends upon detailed analysis of the cancer genome and identification of the targetable aberrations for each individual patient [4]. This approach is substantially limited by the considerable spatial and temporal intratumor heterogeneity of advanced disease. The cancer-related aberrations in the original tumor can differ among tumor regions and distinct disease sites [5].

Molecular testing of tumor samples obtained by surgical procedures or biopsies remains the standard of care [6]. However, this approach has significant limitations because of the temporal and spatial tumor heterogeneity, which would mandate multiple biopsies from primary and metastatic sites at multiple time points. This is not feasible because of the medical condition of patients with advanced cancer, the risk of complications, and various economic and logistic considerations. To overcome these limitations, novel minimally invasive methods to detect pertinent molecular changes in tumors are being developed. Mandel and Métais in 1948 noticed the presence of cell-free nucleic acids (cfNA) in human blood [7]. However, it took several decades before reports emerged on oncogenic mutations in blood-derived cell-free DNA (cfDNA) of patients with cancer [8] or fetal cfDNA in pregnant women [9]. cfDNA also was investigated in prediction of outcome after brain trauma [10], myocardial infarction [11], and stroke [12, 13].

Fragments of cfNA such as DNA, messenger RNA, or microRNA can be detected in plasma, urine, cerebrospinal fluid (CSF), and other body fluids. In cancer patients, these cfDNA fragments can be used for detection of underlying cancer-related molecular abnormalities [8, 14]. Such approaches, which have become known as liquid biopsies, can be used to monitor a cancer molecular profile in real time with minimal invasiveness. It is assumed that fragments of cfDNA are released to blood from diverse tumor sites and perhaps better represent prevailing molecular abnormalities than single-site biopsies. In addition, molecular testing of cfDNA can be used to evaluate response to therapy, disease progression or recurrence, and emergence of molecular abnormalities that drive resistance to systemic therapy.

The Biology of cfDNA

Fragments of cfDNA can be detected in extracellular fluids, such as blood (plasma or serum), urine, CSF, or even ascites, of patients with cancer [15–21], and increased levels of cfDNA can be associated with unfavorable outcome [22, 23]. It has been demonstrated that patients with advanced cancer have higher levels of cfDNA than patients with localized cancer or individuals without cancer [24–34].

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DNA can enter the circulation by several distinct mechanisms, including release of nuclear and mitochondrial DNA from dying cells during either apoptosis or necrosis (Figure 1). Other mechanisms of DNA release include autophagy and necroptosis [35, 36]. cfDNA structural characteristics differ substantially by type of release mechanism. Apoptosis is a programmed and well-controlled process of cellular destruction, and fragments of DNA released from apoptotic cells average around 160–180 bp in length [37, 38]. In contrast, necrosis is a pathological process, and the fragments of DNA are generated more randomly and usually are longer. The average lengths of cfDNA fragments from apoptotic and necrotic processes and their ratio may be assessed as an important element of the DNA integrity index, which may have prognostic implications [39]. Thierry *et al.* described experimental system for studying the cfDNA characteristic based on the nude mice xenografted with human HT29 or SW620 colorectal cancer cells [40]. The discrimination of cfDNA fractions from normal (murine) cells and from *BRAF V600E*-mutated and non-mutated tumor (human) cells was possible and the concentration of tumor (human mutated and nonmutated) but not mouse cfDNA increased significantly with tumor burden ($P>0.001$ and $P<0.05$, respectively). The higher cfDNA fragmentation was also observed in mice with bigger tumors as the integrity index decreased with tumor size. The study confirmed the predominance of mononucleosome-derived cfDNA fragments in plasma from xenografted animals and of apoptosis as a source of tumor cfDNA [40].

It has been proposed that plasma cfDNA can be also involve in the oncogenesis via the uptake of nucleic acids originating from tumor cells by susceptible healthy cells that consequently underwent malignant transformation, the process referred to as “genometastasis” [41, 42]. In the in-vitro study with cultures of NIH-3T3 cells treated with plasma from colorectal cancer patients, the transfer of human DNA were observed and the NIH-3T3 cells were oncogenically transformed, as shown by the development of carcinomas in nonobese diabetic–severe combined immunodeficient mice after the injection of such cells [43].

The cfDNA fragments are cleared from the circulation by the liver and kidney, with half-lives ranging from 15 minutes to a few hours [15, 44].

Technologies for cfDNA Analysis

Sample collection and processing can have significant impacts on cfDNA assessment [45]. Most often the circulating DNA is extracted from plasma; plasma is preferred to serum because of serum has higher levels of non-cancerous cfDNA due to lysis of normal leukocytes. Timely processing is paramount for success [46]. Cell-stabilizing streck tubes, which allow sample processing to be delayed for several days, have become increasingly popular for collection of blood samples intended for cfDNA analysis [47, 48]. Other materials, such urine or CSF, are less cellular and should be less prone to DNA degradation [17, 19]. At the moment, specimen collection protocols vary considerably in details such as use of ethylenediaminetetraacetic acid or even streck tubes.

The optimal pre-analytical handling conditions for the collected blood sample were described by Messaoudi *et al.* [45]. Blood samples must by drawn carefully and agitation

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should be avoided to prevent any hemolysis. The sample may be kept at room temperature or +4 °C and must be processed within 4 h to prevent changes in cfDNA concentration and fragmentation. The two-steps centrifugation (1200–1600 g for 10 min and 16000 g for 10 min) is highly recommended to eliminate any cells from the plasma. The second step can be done after the storage of plasma sample at −20 °C or −80 °C. Plasma as well as cfDNA extracts are sensitive to freeze-thaw cycles. Plasma must be stored at −80 °C up to maximum of nine months before the final cfDNA analysis. The extracts of cfDNA must be stored at −20 °C for up to three months for the concentration and fragmentation analysis or up to nine months for specific mutations analysis [45].

The techniques for the quantification of total amount of cfDNA include fluorescence-based methods (such as Hoechst dye and PicoGreen staining), spectrophotometric-based methods (ultraviolet spectrometry) or quantitative real-time polymerase chain reaction (PCR) methods (such as SYBR Green and TaqMan) [35, 49, 50]. The study with plasma samples collected from 10 non-small cell lung cancer patients compared PicoGreen staining to real-time PCR methods for the quantification of cfDNA [51]. The results from PicoGreen method correlated with both the SYBR Green ($R = 0.87$, $P < 0.0001$) and TaqMan probe approach ($R = 0.94$, $P < 0.0001$). The results from another method for the cfDNA quantification using the fluorescent SYBR Gold staining without prior DNA extraction and amplification showed the high correlation with the conventional quantitative PCR assay of beta-globin ($R = 0.9987$, $P < 0.001$) [52]. Therefore fluorescence-based methods could be the rapid, accurate, and inexpensive alternatives to real-time PCR for total cfDNA quantification.

The tumor-specific fraction of the total cfDNA can be identified by the presence of cancer-specific alterations. Epigenetic modifications such as methylation patterns also are being investigated as signature markers to differentiate tumor-specific cfDNA fraction [53]. The tumor-specific fraction can vary in plasma from 0.01% to more than 90% [36, 54]. Lower-stage tumors have lower levels of cfDNA than advanced disease [28]. Therefore, highly sensitive methods are required for detection of cfDNA in early disease.

Various PCR approaches were used originally to detect tumor-related aberrations in cfDNA. These included methods such as ARMS-Scorpion PCR (amplification refractory mutation system), PCR-SSCP (single-strand conformation polymorphism), ME-PCR (mutant enriched), MASA-PCR (mutant allele–specific amplification), PAP-A amplification (pyrophosphorolysis-activated polymerization allele-specific amplification), or RFLP-PCR (restriction fragment length polymorphism) [55–60]. Even higher sensitivity is required, however, for detection of ctDNA from tumors in which specific mutations occur in very low allele fractions. For this reason, novel methods using digital PCR were introduced into cfDNA assays. Digital PCR methods include droplet-based systems [61], the use of beads, emulsions, amplification, and magnetics (BEAMing) [62], or microfluidic assays [63, 64].

Next-generation sequencing techniques (NGS), which allow detection of multiple alterations across wider regions of the cancer genome, also can be used for testing of cfDNA. The specific regions of cfDNA are analyzed by using targeted deep-sequencing techniques such as TAm-Seq (tagged amplicon deep sequencing) [63], Ion AmpliSeq [65], Safe-Seq (safe-

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sequencing system) [66], or CAPP-seq (cancer personalized profiling by deep sequencing) [67]. The latest and most comprehensive approaches to cfDNA analyses that do not require knowledge of preexisting mutations include whole-exome [68] as well as whole-genome sequencing of plasma samples [69, 70]. The NGS techniques and unbiased whole-exome and whole-genome assays might dominate the future of cfDNA research. The advantages of PCR-based and NGS-based approaches are summarized in Table 1.

Clinical Application of cfDNA in Cancer Management

Identification of molecular targets

The feasibility of identifying molecular targets in cfDNA as well as the level of concordance between mutations detected in tumor tissue and plasma samples are important attributes for future routine clinical use of cfDNA liquid biopsy techniques (Table 2). In a pilot study of 18 patients with metastatic colorectal cancer who were candidates for surgical resection or radiofrequency ablation, oncogenic mutations (*APC*, *TP53, PIK3CA,* and *KRAS*) were assessed by direct sequencing in tumor tissue [15]. At least one mutation was identified in each of the tumors. The unique molecular signature of each tumor was used for detection and quantification of tumor-derived cfDNA by the BEAMing PCR-based technology. This study demonstrated that cfDNA can be isolated from plasma samples and used to identify oncogenic mutations in cancer patients.

In a cohort of 49 patients with advanced breast cancer, there was 100% concordance (34 of 34 cases) between BEAMing-detected *PIK3CA* mutations in plasma cfDNA and in tumor tissues obtained at the same time [62]. However, the concordance decreased to 79% in an additional cohort of 60 patients when tumor samples and plasma cfDNA were obtained at different time points.

In a study of 157 patients with advanced cancer that progressed on systemic therapy who were referred for treatment with experimental targeted therapies, a panel of 21 oncogenic mutations in the *BRAF*, *EGFR*, *KRAS,* and *PIK3CA* genes was assessed in plasma cfDNA by BEAMing technology. The results demonstrated acceptable concordance (*BRAF*, 91%; *EGFR*, 99%; *KRAS*, 83%; *PIK3CA*, 91%) with results of standard-of-care mutation analysis of primary or metastatic tumor tissue obtained during clinical care [71].

Thierry *et al*. [72] assessed the mutation status of *KRAS* and *BRAF* by using allele-specific quantitative PCR of cfDNA in 106 plasma samples from patients with metastatic colorectal cancer and compared it to the mutations detected in tissue (primary or metastatic) tested by standard-of-care methods. The cfDNA analysis showed 100% specificity and sensitivity for the *BRAF V600E* mutation and 98% specificity and 92% sensitivity for the *KRAS* mutations, with a concordance value of 96%.

The *BRAF V600E* mutation was recently detected in plasma and urine cfDNA samples obtained from individuals with Erdheim-Chester disease and Langerhans cell histiocytosis [18]. These patients have a high prevalence of *BRAF V600E* mutations and a good response to BRAF inhibitors. There was 100% concordance between tissue and urinary cfDNA genotypes assessed by droplet-digital PCR assay (ddPCR) in samples from 30 treatment-

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naive patients. The targetable mutation *BRAF V600E* was also analyzed in plasma- and serum-derived cfDNA samples from 221 patients with advanced melanoma [73]. Assay sensitivity for mutation detection was 44% in serum and 52% in plasma. Test specificity was 96% in both matrices.

Panka *et al.* [74] developed blood-based assay for the detection of *BRAF V600E* mutation and used it in the study with 128 patients with stage II-IV melanoma. The high 96% sensitivity and 95% specificity of the assay were observed for the subset of 42 stage IV patients. The area under the receiver operator curve (ROC) was 0.9929 demonstrating an excellent ability to discriminate *BRAF*-mutant melanoma patients. Pupilli *et al.* [75] investigated the role of *BRAF V600E*-mutated allele in plasma cfDNA from 103 patients with papillary thyroid carcinoma (PTC) as a marker for the diagnosis and follow-up. Patients with PTC showed a higher percentage of circulating *BRAF V600E* mutation (P = 0.035) compared to those with benign histology (n=16) and healthy controls (n=49). The assay diagnostic sensitivity and specificity were 80% and 65%, respectively.

Zill *et al.* [76] assessed the mutation status of 54 genes by NGS in the tumor tissue and corresponding cfDNA in plasma samples from 26 patients with pancreatobiliary carcinomas (18 pancreatic ductal adenocarcinoma cases and 8 biliary cancer cases). 90.3% of mutations detected in tumor biopsies were also detected in cfDNA. Across the five most frequently mutated genes in tumor tissue biopsies (*KRAS*, *TP53*, *APC*, *FBXW7* and *SMAD4*), the assay sensitivity for the detection of such mutations in cfDNA was 92.3%, specificity was 100% and the diagnostic accuracy was 97.7%.

Forshew *et al.* [77] reported on using the TAm-Seq method for identification and monitoring of oncogenic mutations in plasma cfDNA. They screened 5995 genomic bases in coding regions of *TP53* and *PTEN* and selected regions in *EGFR*, *BRAF*, *KRAS*, and *PIK3CA* for low-frequency mutations. The assay was able to detect mutations in cfDNA with sensitivity and specificity of >97%. In one patient with synchronous primary cancers of the bowel and ovary, moreover, disease relapse was identified as being derived from the original ovarian tumor. At relapse, analysis of the plasma cfDNA detected the *TP53* mutation (p.R273H) originally found in the ovarian primary tumor, whereas the bowel-associated mutations were not detected.

Beaver *et al.* [61] showed the possibility of identifying *PIK3CA* mutations in plasma samples from 29 patients with early-stage breast cancer. The same mutations identified in primary tumors were detected in pre-surgery plasma samples by ddPCR with high sensitivity and specificity (93.3% and 100%, respectively). Residual disease was successfully identified by detection of the mutations in cfDNA from postoperative plasma samples. In another study of 17 patients with metastatic breast cancer, analysis of primary or metastatic tumors together with matched plasma samples for mutations in 50 selected genes by NGS yielded a concordance of 76% [78].

Bettegowda *et al.* [66] evaluated the possibility to detect the cfDNA point mutations and genetic rearrangements that were originally found in tumor tissue biopsies from 640 patients with various cancer types. Tumor-derived cfDNA was detected in > 75% of patients with

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advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers. In patients with localized tumors, cfDNA was detected in 73, 57, 48, and 50% of patients with colorectal cancer, gastroesophageal cancer, pancreatic cancer, and breast adenocarcinoma, respectively [66].

Newman *et al.* [67] developed CAPP-Seq, an ultrasensitive method for quantifying tumor-derived plasma cfDNA by targeting recurrently mutated regions in the cancer of interest. In patients with non-small cell lung cancer, the CAPP-Seq method was able to detect cfDNA in 100% of patients with stage II–IV disease and 50% of patients with stage I disease. The method specificity was 96% for mutant allele fractions as low as 0.02%.

Assessment of prognosis

The quantification of total and/or mutant cfDNA has been studied for prognosis assessment in various tumor types. Some studies demonstrated that, in cancer patients, higher levels of cfDNA are associated with higher risk of disease recurrence and progression [15, 28, 30, 66, 79–81]. In a study by Diehl *et al.* [15] of 18 colorectal cancer patients, the absence of cfDNA in plasma during the first follow-up visit after surgical resection was associated with 100% recurrence-free survival.

Early limited data suggested that persistence of *TP53* mutation in plasma cfDNA of patients with stage II or III breast cancer that was in remission was associated with higher likelihood of disease recurrence; however, the small sample size precluded any definitive conclusion [56].

The amount of mutant cfDNA has been found to be of prognostic significance. Spindler *et al.* [79] demonstrated the prognostic value of the amount of total cfDNA and *KRAS* mutant cfDNA in a study of 108 patients with metastatic colorectal cancer treated with third-line cetuximab and irinotecan. Patients with higher cfDNA levels had shorter progression-free survival (PFS; 2.1 vs 4.4 months; P=0.0015) and overall survival (OS; 3.6 vs 10.4 months; P<0.0001) than patients with lower cfDNA levels. Similarly, patients with higher levels of *KRAS*-mutant cfDNA had shorter PFS (1.8 vs 2.3 months; P=0.008) and OS (2.1 vs 5 months; P=0.0005) than patients with lower levels of *KRAS*-mutant cfDNA.

The mutated fraction of plasma cfDNA (mutation in codon 12 or 13 of *KRAS)* was assessed in another study of 206 patients with metastatic colorectal cancer [66]. Concentration of mutated cfDNA was found to provide added value in survival prediction (likelihood ratio test, P = 0.00253, df = 3) to the model of well-known prognostic factors (age, Eastern Cooperative Oncology Group performance status, and level of carcinoembryonic antigen). Also, holding other predictors constant, the 2-year survival rate steadily decreased as the plasma concentration of mutated cfDNA increased.

The study already mentioned [71] of the panel of 21 mutations in *BRAF*, *EGFR*, *KRAS,* and *PIK3CA* assessed by BEAMing technology in plasma cfDNA of 157 patients with advanced cancer also examined the prognostic impact of the amount of mutated plasma cfDNA. A higher percentage of mutant cfDNA (>1% [n = 67 patients] vs. 1% [n = 33 patients]), irrespective of type of mutation, was associated with a shorter OS (5.5 vs. 9.8 months; P =

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0.001), which was confirmed in a multivariable analysis. Similarly, 41 patients with >1% of *KRAS* mutant (codon 12 or 13) cfDNA had a shorter median OS than 20 patients with 1% of *KRAS* mutant cfDNA (4.8 vs. 7.3 months; P = 0.008). The significant differences in OS were not observed for mutations in other examined genes, probably because of the smaller sample size.

In another study of 246 patients with advanced non-small-cell lung carcinoma treated with platinum and vinorelbine chemotherapy, the patients with detectable plasma *KRAS* mutant (codon 12 or 13) cfDNA had a shorter median OS (4.8 vs 9.5 months; P = 0.0002) and shorter median PFS (3.0 vs 5.6 months; P = 0.0043) than patients whose cancer expressed wild-type *KRAS* [80]. A multivariate analysis confirmed the independent prognostic value of *KRAS* mutant cfDNA in OS but not in PFS. Wang *et al.* [82] showed the negative prognostic effect of *KRAS* mutation (codon 12 or 13) in plasma cfDNA of 273 patients with advanced non–small cell lung cancer. The median PFS of patients with a plasma *KRAS* mutation was 2.5 months, while that of patients with wild-type *KRAS* was 8.8 months (P < 0.001).

In a study of 44 pancreatic cancer patients, the 1-year survival rate was 0% in those with *KRAS* codon-12 mutation in cfDNA and 24% in those with *KRAS* wild-type in cfDNA (P<0.005), and plasma *KRAS* mutation was the only independent prognostic factor (odds ratio, 1.51; 95% confidence interval [CI], 1.02 to 2.23) [60]. In 103 patients with melanoma receiving biochemotherapy [83], those with a *BRAF* mutation in serum cfDNA had significantly shorter OS than those that did not have the *BRAF* mutation in serum cfDNA (13 vs. 30.6 months, P = 0.039).

The negative prognostic impact of increased levels of mutant cfDNA was supported by other studies in breast cancer [84], colorectal cancer [85, 86], ovarian cancer [87], and other tumor types. Furthermore, the presence of other tumor-related genomic cfDNA aberrations was associated with poor prognosis. Detection of loss of heterozygosity and microsatellite instability in cfDNA was associated with worse prognosis for patients with breast cancer [88], ovarian cancer [89], melanoma [90], lung cancer [91], or other tumor types.

Epigenetic alterations detected in cfDNA can also help determine patient prognosis. The aberrant DNA methylations were detected in cfDNA of patients with breast, lung as well as liver cancer [92–94]. Hypermethylated promoter regions of *BRCA1* in serum cfDNA from 100 primary invasive ductal breast cancer patients was associated with poor DFS (14.2 months; P 0.0001) as well as poor OS (24.3 months; P = 0.0001) [95]. Similarly cfDNA promoter hypermethylation of *GSTP1* was associated with poor DFS (24.2 months; P = 0.03). Another study with 336 primary invasive breast cancer patients showed worse OS rate at 100 months (78 vs. 95%; P = 0.002) for patients with serum cfDNA hypermethylation in promoter regions of *GSTP1*, *RASSF1A*, and *RARβ2* than those with negative findings [96]. In the study with 428 primary breast cancer patients, the detection of methylated *PITX2* and *RASSF1A* in plasma cfDNA determined shorter OS in multivariate analysis (low vs. high methylation; HR 3.4, P = 0.021 and HR 3.4, P = 0.002 respectively) [97]. For distant DFS only *RASSF1A* showed prognostic significance (low vs. high methylation; HR 3.4, P = 0.002). The aberrant methylation of selected genes in cfDNA were associated with poor

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prognosis also in colorectal cancer [98], gastric cancer [99], hepatocellular carcinoma [100] and other tumor types.

Prediction of response to therapy

The liquid biopsy could provide an easy way to assess predictive biomarkers for targeted therapy as well as a minimally invasive way to monitor therapy response in real time [8, 14] (Table 3).

In a prospective study of 52 patients with metastatic breast cancer, the plasma cfDNA was monitored to qualitatively and quantitatively assess disease progression and treatment response and compare with levels of circulating tumor cells (CTC) and tumor marker cancer antigen 15-3 (CA15-3) and computed tomography (CT) imaging [63]. The cfDNA was detected by identification of the same *PIK3CA* and *TP53* mutations and structural variations as were found in the tumor tissues. The levels of cfDNA in plasma generally correlated well with the treatment response assessed by CT imaging (as defined by Response Evaluation Criteria in Solid Tumors). However, two patients in this study had discordant correlations. In 10 of the 19 patients who experienced disease progression, the cfDNA levels increased at one or more consecutive time points, on average 5 months before progressive disease was observed on imaging. Moreover, the cfDNA was found to be a more accurate biomarker for monitoring metastatic disease than CTCs, CA 15-3, or CT imaging.

Another study in 72 patients with advanced non-small cell lung cancer examined the dynamic changes in cfDNA *EGFR* mutations as a predictor of response to EGFR tyrosine-kinase inhibitor (EGFR-TKI) targeted therapy [101]. Failure to clear plasma *EGFR* mutations after EGFR-TKI was an independent predictor for shorter PFS (hazard ratio [HR] 1.97, P = 0.001) and OS (HR 1.82, P = 0.036). The *EGFR* mutations were detected by ddPCR in serial plasma samples of non-small cell lung cancer patients treated with erlotinib [102]. The study demonstrated the disappearance of *EGFR* mutations in exon 19 and 21 and the emergence of *EGFR T790M* resistance mutation several weeks before radiographic disease progression.

Similarly, *EGFR* mutations were detected in primary tumors and corresponding plasma samples in a study of 1060 patients with advanced lung cancer treated with gefitinib [103]. Objective response rates were 76.9% (95% CI, 65.4–85.5) for patients with detected mutations in both tumor and plasma and 59.5% (95% CI, 43.5–73.7) for patients with mutation in the tumor but not in plasma. Median PFS was 9.7 months (95% CI, 8.5–11.0) for patients with mutation in the tumor sample only and 10.2 months (95% CI, 8.5–12.5) for patients with mutation in both tumor and plasma samples. This demonstrated that *EGFR* mutation status could be assessed in cfDNA and serve as a positive predictive biomarker for targeted therapy.

Another study [104] assessed *BRAF* mutations in plasma cfDNA from 160 patients with advanced cancer and known *BRAF* status from archival tumor samples. Patients whose formalin-fixed paraffin-embedded (FFPE) tumor had a *BRAF V600* mutation (n=51) received therapy with a BRAF and/or MEK inhibitor. The time to treatment failure (TTF) of 13 patients with a *BRAF V600* mutation in the tumor but not in plasma obtained before

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therapy was significantly longer than that of 38 patients whose baseline plasma cfDNA had a *BRAF V600* mutation (13.1 vs. 3.0 months; P=0.001). The absence of *BRAF V600*–mutant cfDNA also was associated with longer TTF (HR, 0.31; P=0.004) in multivariate analysis.

Detection of resistance to targeted therapy

The implementation of personalized medicine principles and targeted therapy into routine oncology practice is bringing an important shift in the treatment of advanced cancers. In metastatic disease, a chronic course is no longer unusual, and patients can survive for many years [105]. However, despite the significant initial therapeutic effect of targeted therapy, the vast majority of patients eventually develop resistance and experience tumor progression. The tumor resistance results from acquisition of mutations in the targeted genes or signaling pathways of cancer cells under therapeutic selective pressure (i.e., secondary resistance). The mutations causing resistance also can be present in the infrequent subclones of pretreatment tumor cells and can predict the further failure of targeted therapy (i.e., primary resistance) [5, 14, 106].

The mechanisms of resistance are often known; however, since routine multiple sequential biopsies are not performed, we have no tools to describe these mechanisms at the level of an individual patient. Both intrinsic and adaptive resistance can occur because of pre-existing or acquired molecular abnormalities, such as gatekeeper mutations in the *BCR-ABL* kinase domain, which cause resistance to imatinib and other TKIs in chronic myelogenous leukemia [107]. Similarly, emergence of *KRAS* mutations plausibly causes resistance to EGFR monoclonal antibodies in metastatic colorectal cancer [108], and emergence of *EGFR T790M* mutation causes resistance to EGFR-TKIs in non-small cell lung cancer [109, 110]. Last but not least, *ALK* mutation *L1196M* or *C1156Y* mediates adaptive resistance to crizotinib in non-small cell lung cancer with *ALK* rearrangement [111], and mutations in *NRAS*, *MEK,* and *BRAF* amplification indicate resistance to *BRAF* inhibitor vemurafenib in *BRAF*-mutant melanoma [112]. Because liquid biopsies can be obtained at low cost at multiple time points, they offer a useful tool for monitoring molecular changes associated with resistance to certain cancer therapies (Table 4).

For instance, PCR detection with the BEAMing approach in patients with advanced non-small cell lung cancer demonstrated *EGFR T790M* mutation in cfDNA from 10 of 23 patients who experienced disease progression while receiving an EGFR-TKI [113]. In a different study, digital PCR detection of the *EGFR T790M* resistance mutation in pretreatment cfDNA plasma samples from 135 patients with advanced non-small cell lung cancer treated with an EGFR-TKI was associated with a shorter median PFS (8.9 vs. 12.1 months; P = 0.007) and OS (19.3 vs. 31.9 months; P = 0.001) than no *T790M* mutation [114].

Another example of emerging resistance mutations to targeted therapy with high clinical relevance is the acquisition of tumor *KRAS* mutations in codon 12, 13, or 61 in patients with advanced colorectal cancer treated with anti-EGFR monoclonal antibodies cetuximab or panitumumab [16, 20]. Two landmark studies have shown the possibility of detecting and monitoring these emerging *KRAS* mutations in such patients in cfDNA by using BEAMing technology [16, 20]. Testing of serum cfDNA from 28 colorectal cancer patients receiving

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panitumumab showed that 9 of 24 patients whose tumor and cfDNA were initially *KRAS* wild-type had developed detectable cfDNA *KRAS* mutations [16]. Interestingly, multiple *KRAS* cfDNA mutations were detected in three individuals. The appearance of mutations generally occurred between 5 and 6 months following initiation of treatment. In the second study, emergence of *KRAS* aberrations was found in tumor tissue samples from metastatic sites obtained after initiation of therapy [20]. Corresponding plasma samples also showed emergence of *KRAS* mutation in cfDNA, which could have happened as early as 10 months before radiographic progression [20]. Furthermore, a group from MD Anderson Cancer Center, using BEAMing technology, reported acquired *KRAS* and/or *EGFR* ectodomain mutations in 44% (27/62) and 8% (5/62) of plasma samples from patients with advanced colorectal cancer treated with cetuximab or panitumumab, respectively [115]. *KRAS* codon 61 and 146 mutations were predominant (33% and 11%, respectively).

Even if the candidate-gene techniques to monitor emerging resistance mutations to various targeted therapeutics provide promising results, such approaches have substantial drawbacks, most notably the requirement for prior knowledge of mechanisms of resistance and corresponding mutations. Application of unbiased approaches for detection of emergence of resistant cancer cell subclones using NGS technologies directly on the plasma samples could overcome these limitations. A proof-of-principle study by Murtaza *et al.* [68] monitored cancer clonal evolution and the acquisition of secondary resistance mutations to various anticancer treatments in serial plasma samples from six patients with advanced breast, ovarian, or lung cancer using unbiased whole-exome sequencing. Follow-up intervals were 1–2 years, and the exome sequencing was performed on two to five plasma samples in each patient. The results revealed emergence of distinct secondary mutations, such as an activating mutation in *PIK3CA* after paclitaxel, a truncating mutation in *RB1* after cisplatin, a truncating mutation in *MED1* after tamoxifen and trastuzumab and a splicing mutation in *GAS6* after subsequent treatment with lapatinib in the same patient, and a *T790M EGFR* mutation after treatment with gefitinib. The results of this study established that exome-wide analysis of cfDNA could complement standard biopsy to detect mutations associated with acquired resistance to therapeutic agents in advanced cancers. However, it should be noted that the detected mutant allele fractions for the aberrations were rather high (3%–45%), which can limit the applicability of such an approach to a limited subset of patients.

Overall, liquid biopsy–guided detection of clonal evolution and acquired mechanisms of resistance can be an attractive tool in cancer therapy. However, its utility needs to be tested in future prospective clinical trials that use liquid biopsies as a tool for therapeutic decision making (Table 5).

Expert Commentary

Liquid biopsy utilizing cfDNA is an attractive tool in oncology for identification of molecular targets, determination of prognosis, assessment of response to anticancer therapy, and real-time monitoring of cancer molecular profile. However, the clinical utility of molecular profiling in cfDNA remains to be proven in prospective studies. Retrospective observations demonstrated that changes in the amount of mutant cfDNA can indicate response to anticancer therapy and that emergence of certain molecular abnormalities can

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predict emergent therapeutic resistance; however, it will remain unclear whether this offers any clinical advantage or alters therapeutic decisions until it is tested in prospective controlled clinical trials. Even though most cfDNA technologies have demonstrated high concordance with molecular testing of tumor tissue, there is still uncertainty whether molecular profile from cfDNA can replace tissue testing, at least in situations when the tissue is in short supply. Furthermore, cfDNA consists of both nonmalignant and tumor DNA, unlike tumor tissue, increasing the need for high sensitivity and limiting the use of technologies such as whole-genome or -exome NGS. Also, cfDNA occurs in short fragments, which can further complicate molecular analysis.

Five-year View

In the next 5 years, cfDNA-based liquid biopsies will be implemented in clinical studies and drug development. Such studies will provide real-time evaluation of pertinent biomarkers as well as the technology itself. Liquid biopsy approaches have been selected for testing as exploratory endpoints in national molecular matching initiatives such as the multi-arm NCI MATCH clinical trial. Furthermore, randomized studies exploring whether liquid biopsy approaches can be used for biomarker detection and subsequent treatment allocation in lieu of tumor tissue are being designed. Liquid biopsies will likely become an integral part of diagnostics in oncology; however, they are not expected to entirely replace tumor biopsies since they cannot address many important factors such as changes in and interactions with the tumor microenvironment. Furthermore, novel liquid sources of DNA will be tested and validated, including CTC and exosomes [116–120].

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List of abbreviations

ABL	abelson murine leukemia viral oncogene homolog;
ALK	anaplastic lymphoma kinase
APC	adenomatous polyposis coli gene
ARMS	amplification refractory mutation system
BCR	breakpoint cluster region
BEAMing	beads emulsions, amplification and magnetics
BRAF	v-raf murine sarcoma viral oncogene homologe B1
CA 15-3	cancer antigen 15-3
CAPP-seq	cancer personalized profiling by deep sequencing
CEA	carcinoembryonic antigen

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cfDNA	cell-free DNA
cfNA	cell-free nucleic acids
CSF	cerebrospinal fluid
CT	computed tomography
CTC	circulating tumor cells
ddPCR	droplet digital polymerase chain reaction
EGFR	epidermal growth factor receptor
FFPE	formalin-fixed paraffin-embedded
GAS6	growth arrest-specific 6
KRAS	kirsten rat sarcoma viral oncogene homolog
MAPK	mitogen-activated protein kinase
MASA	mutant allele specific amplification
ME	mutant enriched
MED1	mediator complex subunit 1
MEK	mitogen-activated protein kinase kinase
NGS	next generation sequencing techniques
NRAS	neuroblastoma RAS viral oncogene homolog
OS	overall survival
PAP-A	pyrophosphorolysis-activated polymerization-allele-specific amplification
PCR	polymerase chain reaction
PFS	progression-free survival
PI3K	phosphatidylinositol 3-kinase
PIK3CA	catalytic domain p110α of the class I phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
RB1	retinoblastoma gene
RFLP	restriction fragment length polymorphism
Safe-Seq	safe-sequencing system
SSCP	single-strand conformation polymorphism
TAm-Seq	tagged amplicon deep sequencing

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TKI tyrosine-kinase inhibitor

TP53 tumor protein p53

TTF time to treatment failure

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Key issues

- Identification of oncogenic aberrations provided key insight into cancer biology and led to discovery of new targeted therapies.
- Tumor-specific aberrations are usually tested in archival tumor tissue, and limitations or absence of such tissues can preclude molecular analysis and limit the use of personalized therapy.
- Small fragments of cancer cell–free DNA are released into the circulation and can be detected in blood, urine, or other biologic materials.
- Testing for oncogenic mutations in cell-free DNA, which is not all from the tumor, requires highly sensitive methods capable of detecting one mutant allele in 1,000–10,000 wild-type background alleles.
- PCR-based technologies are highly sensitive but do not allow testing for a broad spectrum of aberrations in cell-free DNA. Next-generation sequencing can detect multiple aberrations, but with somewhat lower sensitivity than PCR.
- Detection of oncogenic aberrations in cell-free DNA demonstrated high though not absolute concordance with tumor tissue and can be used plausibly for treatment selection.
- Quantity of mutant cell-free DNA seems to be of prognostic value in predicting survival.
- Dynamic tracking of molecular aberrations in cell-free DNA has potential to be used for monitoring of treatment response in lieu of standard imaging.
- Emergence of molecular aberrations in cell-free DNA can provide insight into mechanism of resistance at the individual patient level and can be investigated as a plausible tool for treatment guidance.

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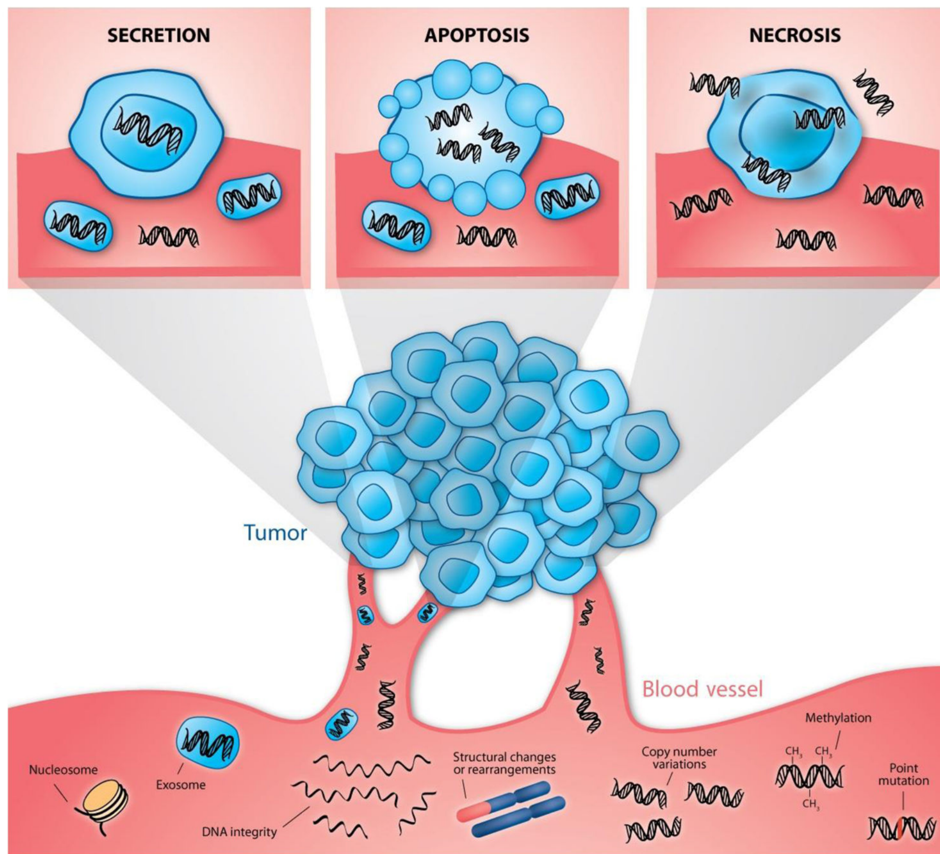

Figure 1.

Passive (from apoptotic and necrotic cells) and active release of DNA fragments from tumor cells into the circulation. This cell-free DNA can be used for testing of tumor-specific aberrations.

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Table 1

Comparison of PCR-based and next genration sequencing-based approaches for cfDNA analysis and their possible applications

Methods	PCR - based approaches	Next-generation sequencing
Important features	Sensitive	Less sensitive in most cases
	Short turnaround	Longer turnaround times
	Tested and validated	Tested and validated
	Available in the CLIA-certified environment	Available in the CLIA-certified environment
	Limitations in terms of multiplexing	Need for bioinformatics, higher cost
Possible applications	Molecular diagnosis in well-defined situation when limited mutation panel is adequate	Molecular diagnosis in clinical setting
	Monitoring of mutation load as a surrogate for response to therapy	Discovery research
	Diagnosis of emergent resistance clones in malignancies with well-defined mechanisms of resistance	Malignancies with undefined patterns of resistance to targeted therapy

Abbreviati ons: CLIA, clinical laboratory improvement amendments; PCR, polymerase chain reaction

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Table 2

The summarization of the studies that examined the concordance between mutations detected in tumor tissue samples and cfDNA

Cancer type	Mutated genes	Cohort no.	Results of the concordance between cfDNA and tumor tissue	Reference
Colorectal cancer	*KRAS, BRAF*	106	100% specificity and sensitivity for the *BRAFV600E* mutation and 98% specificity and 92% sensitivity for the *KRAS* mutations detection	Thierry *et. al.* (2014)
Breast cancer	*PIK3CA*	49 + 60	100% concordance if tumor and cfDNA samples collected at the same, 79% in another cohort if collected at different time points	Higgins *et al.* (2012)
	PIK3CA	29	93.3% sensitivity and 100% specificity for the mutations detection in pre-surgery plasma samples	Beaver *et al.* (2014)
	50 selected genes (NGS)	17	76% concordance between the analysis of primary or metastatic tumors and matched plasma samples	Rothe *et al.* (2014)
Melanoma	*BRAF*	221	Sensitivity for *BRAFV600E* mutation detection in serum was 44%, in plasma 52%. Specificity was 96% in both matrices	Aung *et al.* (2014)
	BRAF	128	96% sensitivity and 95% specificity for *BRAFV600E* mutation detection in the subset of 42 stage IV patients	Panka *et al.* (2014)
Pancreatobiliary carcinomas	54 selected genes (NGS)	26	90.3% of mutations detected in tumor biopsies were also detected in cfDNA. 92.3% sensitivity and 100% specificity across the five most frequently mutated genes	Zill *et al.* (2015)
ECD/LCH	*BRAF*	30	100% concordance of *BRAFV600E* mutation between tissue and urinary cfDNA	Hyman *et al.* (2015)
Advanced cancers	*BRAF, EGFR, KRAS, PIK3CA*	157	Acceptable concordance (*BRAF* , 91%; *EGFR* , 99%; *KRAS* , 83%; *PIK3CA* , 91%) with standard-of-care mutation analysis of primary or metastatic tumor tissue	Janku *et al.* (2015)

Abbreviati ons: NGS, next generati on sequencing techniques; ECD/LCH, Erdheim-Chester disease and Langerhans cell histi ocytosis

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Table 3

Examples of studies that assessed cfDNA for response prediction and/or therapeutic monitoring

Cancer type	Mutated genes	Cohort no.	Therapeutic intevention	Results	Reference
Lung cancer	EGFR	1060	gefitinib	ORR were 76.9% (95% CI, 65.4–85.5) for EGF-mutant tumors and mutated cfDNA, and 59.5% (95% CI, 43.5–73.7) for *EGFR*- mutant tumors and wt cfDNA	Douillard *et al*. (2014)
	EGFR	72	EGFR-TKIs	Failure to clear *EGFR*- mutant cfDNA after EGFR-TKI was an independent predictor of shorter PFS (HR 1.97, P = 0.001) and OS (HR 1.82, P = 0.036)	Tseng *et al*. (2014)
Breast cancer	PIK3CA, TP53, structural variations	52	various systemic therapeutics	The levels of cfDNA in plasma generally correlated well with the treatment response assessed by CT imaging	Dawson *et al*. (2013)
Melanoma	BRAF	160	BRAF and/or MEK inhibitors (n=51)	TTF of 13 patients whose baseline cfDNA samples (but not tissue samples) did not have *BRAFV600* mutation was significantly longer than that of 38 patients with baseline cfDNA-mutated samples (13.1 vs. 3.0 months; P=0.001)	Janku *et al*. (2015)

Abbreviations:CT, computed tomography; HR, hazard ratio; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; TKIs, tyrosine kinase inhibitors; TTF, time to treatment failure; wt, wild type

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Table 4

Examples of studies that assessed cfDNA as the biomarker to monitor resistance to the administrated therapy

Cancer type	Resistance mutation	Cohort no.	Therapeutic intervention	Results	Reference
Lung cancer	*EGFR T790M*	23	EGFR-TKIs	*EGFR T790M* mutation was detected in 10 of 23 patients with progression on EGFR-TKI	Taniguchi *et al*. (2011)
	pretreatment *EGFR T790M*	135	erlotinib, gefitinib	Pretreatment detection of mutation in plasma cfDNA predicted lower PFS (8.9 vs. 12.1 months, p =0.007) and OS (19.3 vs. 31.9 months, p = 0.001) compared to patients without mutation	Wang *et a*l. (2014)
Colorectal cancer	*KRAS* mutations	28	panitumumab	Serum cfDNA *KRAS* mutations were detected in 9 of 24 originally *KRAS* wild-type patients	Diaz *et al*. (2012)
	KRAS and/or	62	cetuximab, panitumumab	Acquired KRAS and/or EGFR ectodomain mutations were	Morelli *et al*. (2015)
	EGFR ectodomain mutations			detected in 44% (27/62) and 8% (5/62) plasma samples after treatment	
Breast, ovarian, and lung cancers	plasma whole- exome sequencing	6	various therapeutics	Activating mutations in *PIK3CA* after paclitaxel, *RB1* after cisplatin, *MED1* after tamoxifen and trastuzumab, and *GAS6* after lapatinib; *T790M EGFR* mutation after gefitinib	Murtaza *et a*l. (2013)

Abbreviations: OS, overall survival; PFS, progression-free survival; TKIs, tyrosine kinase inhibitors

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Table 5

Overview of active clinical trials with cfDNA as their primary or secondary objective

Cancer type	Clinical trials with cfDNA as primary or secondary objective [ClinicalTrials.gov Identifier]			
	Assesment of prognosis	**Prediction and monitoring of response to therapy**	**Detection of disease recurrence**	**Detection of resistance to therapy**
Breast cancer	NCT00899548, NCT02306096	NCT01617915, NCT00899548, NCT02109913, NCT01160211, NCT02318901	NCT01617915, NCT02318901	NCT01884285
Lung cancer	NCT02245100	NCT02169349, NCT01930474, NCT02186236, NCT01884285, NCT02281214	NCT02169349	NCT02169349, NCT01930474, NCT01884285, NCT02281214, NCT00997334
Colorectal cancer	NCT01198743	NCT01983098, NCT01212510, NCT01943786	NCT01983098, NCT01943786, NCT01198743	
Melanoma		NCT02251314, NCT02171286, NCT02071940		NCT02251314, NCT02133222, NCT02071940
Pancreatic cancer	NCT02072616, NCT02331251	NCT02331251	NCT02331251	
Hepatocellular carcinoma	NCT02036216	NCT02036216	NCT02036216	
Gastrointestinal stromal tumor		NCT01462994	NCT02331914	NCT02331914
Lymphoma		NCT02339805	NCT02339805	
Prostate cancer		NCT01884285		NCT01884285
Thyroid cancer		NCT01723202		