

## Circulating Tumor DNA as a Liquid Biopsy for Cancer

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**BACKGROUND:** Targeted therapies have markedly changed the treatment of cancer over the past 10 years. However, almost all tumors acquire resistance to systemic treatment as a result of tumor heterogeneity, clonal evolution, and selection. Although genotyping is the most currently used method for categorizing tumors for clinical decisions, tumor tissues provide only a snapshot, or are often difficult to obtain. To overcome these issues, methods are needed for a rapid, cost-effective, and non-invasive identification of biomarkers at various time points during the course of disease. Because cell-free circulating tumor DNA (ctDNA) is a potential surrogate for the entire tumor genome, the use of ctDNA as a liquid biopsy may help to obtain the genetic follow-up data that are urgently needed.

**CONTENT:** This review includes recent studies exploring the diagnostic, prognostic, and predictive potential of ctDNA as a liquid biopsy in cancer. In addition, it covers biological and technical aspects, including recent advances in the analytical sensitivity and accuracy of DNA analysis as well as hurdles that have to be overcome before implementation into clinical routine.

**SUMMARY:** Although the analysis of ctDNA is a promising area, and despite all efforts to develop suitable tools for a comprehensive analysis of tumor genomes from plasma DNA, the liquid biopsy is not yet routinely used as a clinical application. Harmonization of preanalytical and analytical procedures is needed to provide clinical standards to validate the liquid biopsy as a clinical biomarker in well-designed and sufficiently powered multicenter studies.

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Improvement in the clinical outcome of many cancer types is likely to be achieved by identification of the molecular events that underlie their pathogenesis. With the use of so-called biomarkers, therapies tailored to the genetic composition of tumors are administered. Tumor

genotyping is one possible method of categorizing tumors for clinical decisions and has the potential to identify patients who will likely respond to various drugs.

Recently, substantial progress has been made in the discovery of new biomarkers, such as activating mutations in the epidermal growth factor receptor (*EGFR*)<sup>2</sup> and the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene and amplification of the v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) and the echinoderm microtubule-associated protein like 4–anaplastic lymphoma receptor tyrosine kinase (*EML4–ALK*) fusion gene. Cancers harboring these mutations are responsive to specific targeted inhibitors (1). As large-scale cancer genome sequencing studies illuminate novel cancer-associated mutations at an unprecedented rate, and as our knowledge about functional consequences of these genetic alterations rapidly grows, more biomarkers will emerge.

Although targeted therapies have markedly changed the treatment of cancer over the past 10 years, these therapies have introduced several new issues and challenges, including tumor heterogeneity and molecular evolution, costs and potential morbidity of biopsies, lack of effective drugs against most genomic aberrations, technical limitations of molecular tests, and reimbursement and regulatory hurdles (2). One of the most important biological issues is intratumoral heterogeneity. Almost all tumors treated with any therapy acquire resistance as a result of tumor heterogeneity, clonal evolution, and selection. Gerlinger et al. observed tremendous heterogeneity in multiple tumor suppressor genes (3), which might have an impact, especially on patients with synchronous metastasized cancer, due to the fact that, in most cases, only biopsies are available and treatment decisions depend on the results from a single tumor biopsy. As a consequence, relevant lesions might be overlooked (Fig. 1). Moreover, in most cases biopsies are difficult to obtain and no information about the genetic makeup of metastases is available. Therefore, treatment decisions are often made without any knowledge of the genetic composition of the tumor (Fig. 1). In addition, tumors evolve and subclones

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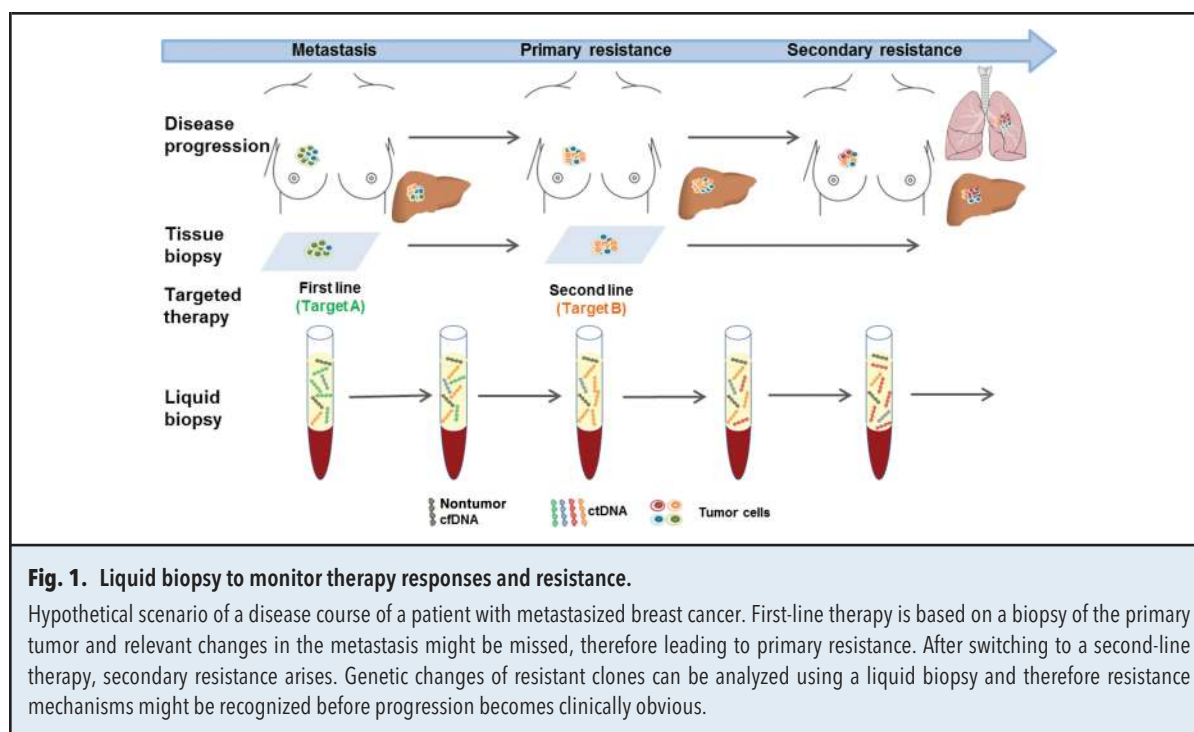
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<sup>2</sup> Human genes: *EGFR*, epidermal growth factor receptor; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *ERBB2*, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; *EML4*, echinoderm microtubule-associated protein like 4; *ALK*, anaplastic lymphoma receptor tyrosine kinase; *SEPT9*, septin 9; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *MET*, MET proto-oncogene, receptor tyrosine kinase; *CDK6*, cyclin-dependent kinase 6; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase.

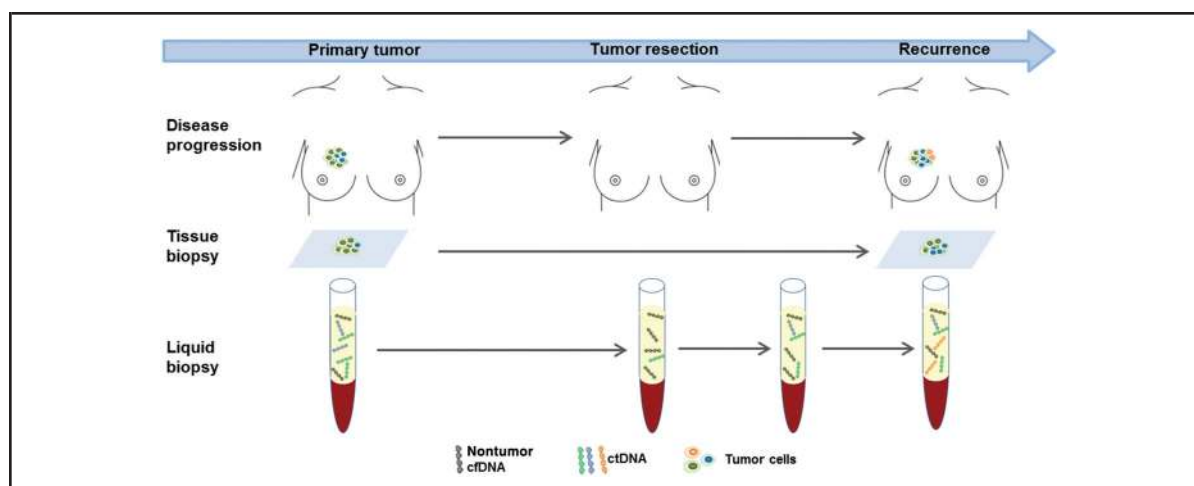


may arise during progression, which results in differences in the proportion and pattern of specific aberrations between the primary tumor and the metastases or relapses (4). Moreover, it has been shown that metastases are not necessarily more complex than the respective primary tumor, e.g., they can lose aberrations that are present in the primary lesion (5) (Fig. 1). With this tremendous heterogeneity and lack of knowledge about the genetic composition of a tumor, the reasons for primary resistance or the development of secondary resistance often remain unknown. Thus, since therapy-related markers may change throughout tumor progression, marker investigations at multiple time points may provide crucial information for patient management (Figs. 1 and 2). Serial sampling of the tumor genome to monitor treatment response should represent a prerequisite for personalized therapy; however, this is currently almost impossible due to the fact that consecutive biopsies are usually a burden for the patient because of the invasiveness of the procedure. In addition, it has been proposed that certain biopsies can confer tumor seeding, although this is considered a very rare event (6). In summary, although invasive procedures give rise to potential risks, the current alternative treatment regime involves an unacceptable level of toxicity and bases progressive disease on clinical parameters that are often not very accurate, i.e., radiological imaging and/or blood measurements.

To overcome these issues, methods will be needed for a rapid, cost-effective, and noninvasive identification

of biomarkers at various time points during the course of disease, i.e., at the time of diagnosis and at defined intervals during treatment. One alternative to overcome the limitation of repeated sampling is the analysis of circulating tumor cells (CTCs)<sup>3</sup> and/or cell-free circulating tumor DNA (ctDNA). Recent progress in ctDNA and CTC analyses now allows the monitoring of tumor genomes by noninvasive means. Multiple studies have shown that it is possible to reconstruct tumor genomes from plasma DNA (7–11). Tumor cells release DNA fragments into the circulation, which can be found in the cell-free fraction of blood together with DNA fragments from normal cells [cell-free DNA (cfDNA)]. Because ctDNA is a potential surrogate for the entire tumor genome, it is often referred to as a “liquid biopsy.” As the trend of optimal therapy management is toward decisions based on the current status of the entire tumor genome, the use of ctDNA as a liquid biopsy may help to obtain the urgently needed genetic follow-up data. This review covers biological and technical aspects as well as

<sup>3</sup> Nonstandard abbreviations: CTC, circulating tumor cell; ctDNA, circulating tumor DNA; cfDNA, cell-free circulating DNA; MSI, microsatellite instability; CRC, colorectal cancer; cfDNA, cell-free fetal DNA; MPS, massive parallel sequencing; EBV, Epstein-Barr virus; dPCR, digital PCR; BEAMing, beads, emulsions, amplification, and magnetics; CAPP-Seq, cancer personalized profiling by deep sequencing; Safe-SeqS, Safe-Sequencing System; TCGA, The Cancer Genome Atlas; TAM-Seq, tagged-amplicon deep sequencing; CNA, copy number aberration; PARE, personalized analysis of rearranged ends; LOH, loss of heterozygosity.



**Fig. 2. Liquid biopsy to monitor tumor-specific aberrations to detect recurrence.**

Hypothetical scenario of a disease course of a breast cancer patient. After curative resection of the primary tumor, there may be no clinical evidence of disease for a long period of time. However, tumor-specific DNA might be detected in the circulation long before recurrence of the tumor is clinically noted. In addition, tumor-specific changes may be used to identify patients with a high risk of recurrence.

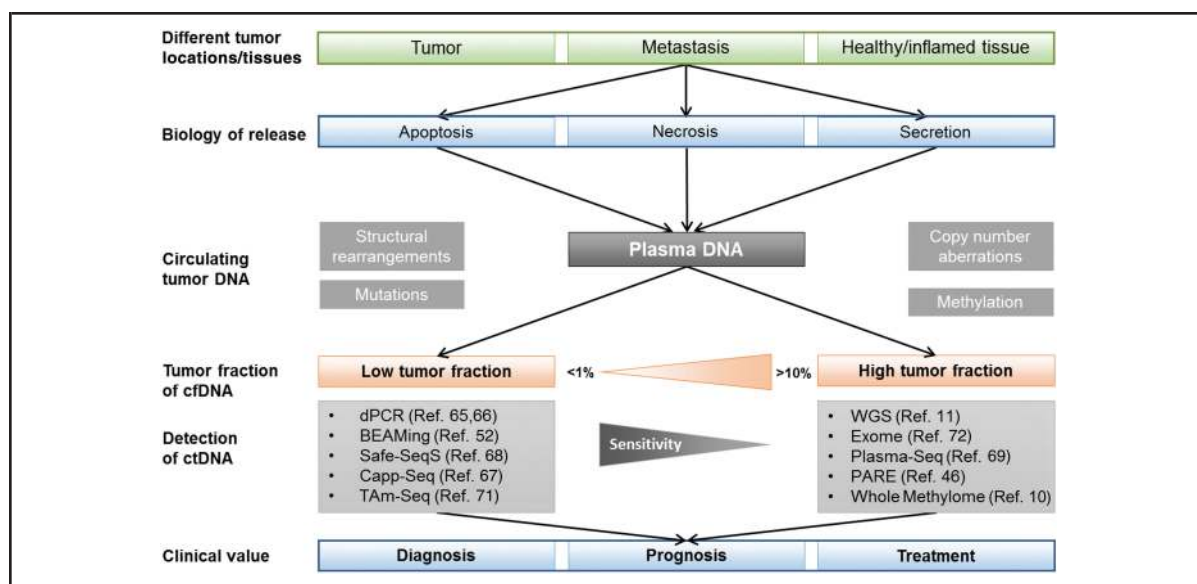
challenges for a widespread implication of cfDNA in cancer diagnostics. As this is a truly growing field and the data on cfDNA are extensive, we were not able to include all existing studies regarding this particular field.

### Biological Aspects

cfDNA was initially identified by Mandel and Métais in the blood of healthy individuals (12). However, their pioneering work did not arouse much interest and it took 30 years until Leon et al. reported increased concentrations of cfDNA in the circulation of cancer patients (13). It took another 10 years until Stroun et al. demonstrated the presence of neoplastic characteristics in the circulation (14). These findings were then confirmed by several other groups, and in the following years tumor-specific aberrations, including mutations in tumor suppressors and oncogenes (15), microsatellite instability (MSI) (16), and DNA methylation (17), were identified and provided concrete evidence that cfDNA is released into the circulation by tumors (Fig. 3).

There are few data available on the actual kinetics of cfDNA release in the circulation, and knowledge of its origin, mechanism, and rate of release is often contradictory. cfDNA is thought to originate from different sources, including apoptotic and necrotic cells (18) (Fig. 3). Some reports indicate that malignancy of the tumor leads to a higher degree of necrosis, corresponding to an increase in circulating tumor DNA. Diehl et al. suggested that DNA fragments found in the circulation are derived from necrotic neoplastic cells that had been engulfed by macrophages (19). In a recent study from Sikora et al.,

larger necrosis-derived DNA fragments were barely detectable in patients with pancreatic ductal adenocarcinoma, pancreatic neuroendocrine tumor, or chronic pancreatitis (20). Alternatively, it has been suggested that all living cells actively release DNA into the circulation (21). However, using whole genome sequencing of plasma DNA of pregnant women, Lo et al. demonstrated that plasma DNA molecules showed a predictable fragmentation pattern reminiscent of nuclease-cleaved nucleosomes (22). This was also confirmed by assessment of the size distribution of cfDNA in healthy individuals and cancer patients, which revealed an enrichment of fragments in the size of single or multiples of nucleoprotein complexes (23) and suggested that the main driver of release may be apoptosis (Fig. 4). Further evidence for apoptosis as the major source of cfDNA came from mice experiments showing that the predominant fragments in plasma from xenografted animals were mononucleosome derived (24). The authors demonstrated that ctDNA features vary during colorectal cancer (CRC) tumor development in nude mice that were xenografted with the human CRC cell lines HT29 or SW620 (24). Although ctDNA was already detectable at an early stage, the size of the resulting tumors did not significantly correlate with the concentration of detectable cfDNA. A thorough investigation of cfDNA size distribution from Mouliere et al. showed that tumor-derived ctDNA in plasma samples from xenografted mice and cancer exhibited a specific profile based on ctDNA size and significantly higher ctDNA fragmentation (25). This was also confirmed by a study from Garcia-Olmo et al. in which they demonstrated that the release of normal



**Fig. 3.** Schematic representation of the liquid biopsy as a tool for cancer monitoring.

Cell-free DNA is released from different tumor locations and healthy/inflamed tissue through multiple mechanisms from cells undergoing apoptosis or necrosis. Tumor-derived DNA (ctDNA) can be extracted from plasma and a variety of tumor-specific genetic changes can be detected. However, the amount of tumor-specific DNA can greatly vary (<1% to >90%) and numerous techniques for the analysis of ctDNA have been proposed. The liquid biopsy may find its clinical application in various clinical settings, including cancer diagnosis, detection of minimal residual disease, prognosis, and therapy monitoring [Taly et al. (65), Vogelstein and Kinsler (66), Diehl et al. (52), Kinde et al. (68), Newman et al. (67), Forshew et al. (71), Chan et al. (11), Murtaza et al. (72), Heitzer et al. (69), Leary et al. (46), Chan et al. (10)]. WGS, whole genome sequencing.

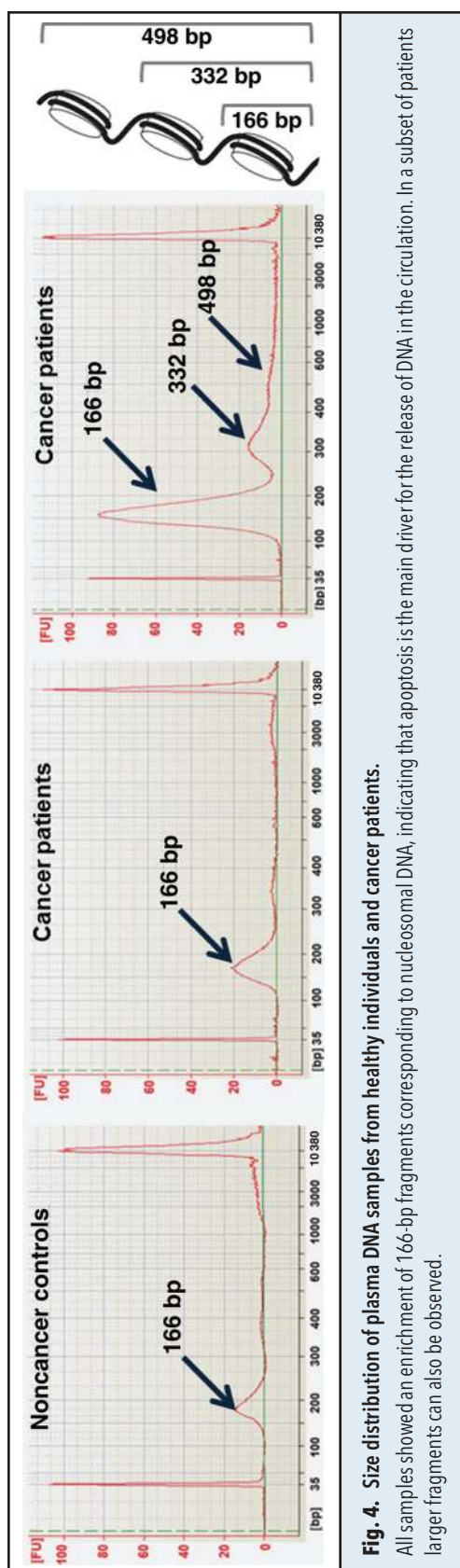
and tumor DNA into plasma appeared to be related to individual-specific factors and that the contribution of tumor DNA to the concentrations of both mutated and nonmutated DNA fluctuated with time (26). The same group proposed that cfDNA in the plasma may participate in tumorigenesis and the development of metastases via transfection-like uptake of such nucleic acids by susceptible cells. Supplementation of plasma from patients with *KRAS* mutations led to oncogenic transformation of murine NIH-3T3, a process called genometastasis (27). A recent study presented the first visual determination of cfDNA by atomic force microscopy on plasma samples from CRC patients and healthy donors, and again showed that more than 80% of cfDNA fragments in CRC plasma were below 145 bp, confirming the high degree of fragmentation through apoptotic mechanisms (28).

Furthermore, there are fewer data available on the stability of cfDNA; however, clearance mechanisms seem to be rapid and the spleen, liver, and kidneys may be responsible for clearance (10, 29). The half-life of cell-free fetal DNA (cffDNA) was previously estimated to 16 min (30); however, a recent study from the same group used massive parallel sequencing (MPS) to study the kinetics of cffDNA, which revealed a biphasic clearance

with half-lives of about 1 h for the rapid phase and a second phase of 13 h (29). Using MPS for assessment of release and clearance dynamics of cfDNA is beneficial to targeted approaches for 2 reasons. First, the entire genome can be used and therefore, MPS does not depend on DNA fragments harboring specific genomic loci. Second, MPS allows the detection of all circulating DNA fragments independent of size (29). Nevertheless, in cancer patients the mechanism of DNA clearance from plasma is poorly understood and it is not known how other factors such as circadian rhythms, inflammation, or particular therapies influence release and clearance mechanisms. The first evidence for equivalent mechanisms was shown in studies with circulating Epstein–Barr virus (EBV) DNA (31, 32).

### Technical Aspects

Owing to the high degree of fragmentation and its low concentration in the circulation, cfDNA is a challenging analyte. Nevertheless, plasma has been shown to be a better source than serum for cfDNA analysis, although the amount of cfDNA in serum can be 2–24 times higher than in plasma (33). This can mainly be attributed to contamination from cells during the clotting process, and



for this reason many laboratories recommend the use of plasma as a source for the analysis of tumor-specific DNA due to lower concentrations of background wild-type DNA. Although the vast majority of studies show higher concentrations of cfDNA in cancer, it has not yet been possible to evaluate the extent to which cfDNA in the circulation of a patient is cancer specific, owing to the fact that increased concentrations of cfDNA have also been detected in physiological and noncancerous pathological conditions. In contrast, when comparing data from plasma and serum samples, an apparent overlap has been observed between healthy individuals and cancer patients (34).

It still seems that a higher amount of cfDNA tends to reflect the *in vivo* situation in cancer patients. Before considering cfDNA concentrations as an informative diagnostic biomarker, a variety of issues need to be solved. First, preanalytical procedures need to be standardized. There are several studies dealing with blood processing methods, including the time elapsed between receiving the sample and the start of the isolation procedure, the centrifugation conditions, and whether serum or plasma is used (35). Selection of an isolation method that ensures extraction of a sufficient amount of high-quality DNA is critical and it has been shown that preanalytical factors of blood sampling and processing can strongly affect DNA yield (36). Because many conventional methods for the isolation of cfDNA are costly, time-consuming, and complex, Sonnenberg et al. developed a novel electrokinetic technique that allowed rapid isolation of cfDNA directly from blood (37). Second, one of the most important issues is the lack of harmonization of quantification methods. Different quantification methods, including spectrophotometric methods, fluorescent dyes, or quantitative PCR-based methods produce different results because these measurements target either total or only amplifiable DNA (38). Recently, Devonshire et al. compared quantitative PCR measurements of 7 different reference gene assays. They observed that loci adjacent to telomeres were more abundant than those with a more centromeric position, demonstrating that the measurement of single gene loci was prone to biases. This is especially true for the quantification of ctDNA, because a variety of copy number changes occur in a tumor (38). Other approaches rely on the quantification of cfDNA directly from plasma without any prior DNA isolation (39). Third, less is known about the origin and the detailed mechanism of cfDNA release, and in most studies confounding events that might also contribute to the release of cfDNA, e.g., nonmalignant diseases, heavy smoking, pregnancy, exercise, and heart dysfunction, have not been taken into account.

Therefore, a consensus on reliable and efficient methods for cfDNA quantification and analysis is essential for the clinical evaluation of ctDNA as a liquid biopsy

**Table 1. Clinical applications of the liquid biopsy.**

Approach	Application	Reference
Diagnostic	Early detection	Sozzi et al. (40), Kim et al. (41), Chen et al. (42), Sozzi et al. (43), Madhavan et al. (44), Frattini et al. (45)
	Monitoring of minimal residual disease	Chan et al. (11), Flohr et al. (47), McBride et al. (48), Shaw et al. (73)
Predictive	Assessment of molecular heterogeneity of overall disease	Chan et al. (10), Chan et al. (11), Leary et al. (46)
	Monitoring of tumor dynamics	Diehl et al. (52), Dawson et al. (54), Heitzer et al. (69), Shaw et al. (73)
	Identification of genetic determinants for targeted therapy	Karapetis et al. (57), Kuo et al. (58)
	Evaluation of early treatment response	Thierry et al. (8), Valtorta et al. (61)
	Assessment of evolution of resistance in real time	Mohan et al. (60), Valtorta et al. (61), Misale et al. (62)
Prognostic	Identification of high risk of recurrence	Lecomte et al. (51), Shaw et al. (73)
	Correlation with changes in tumor burden	McBride et al. (48), Spindler et al. (53), Valtorta et al. (61), Schmitt et al. (74)

to obtain more consistent data that can be compared in different laboratories.

### Clinical Use of Liquid Biopsies

Applications of the liquid biopsy in the clinic are manifold because the concept of a liquid biopsy using blood represents a promising tool to track down tumor-specific changes during the entire course of the disease (Fig. 3, Table 1). A liquid biopsy can be used to identify surrogate indicators for disease recurrence as well as disease progression and can indicate if a specific treatment is applicable or will reduce the risk of recurrence or progression.

#### cfDNA AS A DIAGNOSTIC BIOMARKER

The first efforts toward the clinical use of cfDNA as a liquid biopsy concentrated on simple quantitative assessment of DNA concentrations present in the circulation. Several reported studies showed significant differences in the amounts of plasma DNA isolated from healthy individuals, patients with benign disease, and cancer patients. Although some studies revealed significantly higher concentrations of cfDNA in cancer patients and that simple quantification of cfDNA can confirm the presence of cancer or disease-free status and relapse after curative surgery (40, 41), numerous other studies demonstrate that solely the amount of cfDNA is not a useful diagnostic tool and that the utility of cfDNA is limited without knowledge of tumor mutations (42, 43). A study that analyzed total plasma DNA concentrations and tumor-specific *KRAS* mutations in CRC patients showed that a

higher amount of tumor-specific fragments and that a higher number of CTCs were linked to biphasic size distributions of plasma DNA fragments (Fig. 4). However, despite advanced tumor stage, not all patients had detectable concentrations of ctDNA in their circulation (23). This was confirmed by a recent study from Bettgowda et al. (7). Madhavan et al. evaluated the integrity of cfDNA in a large cohort of breast cancer patients ( $n = 383$ ) and a set of healthy controls ( $n = 100$ ). A hierarchical decrease in cfDNA integrity and an increase in cfDNA concentration from healthy controls to patients with localized diseases to metastatic breast cancer patients was observed (44). Another study showed high concentrations of cfDNA both in plasma and serum (quantitative approach) at the time of surgery in all analyzed CRC patients, with altered carcinoembryonic antigen values in only about 37% of cases (45).

Although simple quantification of cfDNA might not be useful for diagnosis and estimation of prognosis, monitoring of tumor-specific changes may be used as a tool for early cancer detection and/or prognosis. However, the fact that the fraction of circulating DNA that is derived from the tumor can range between 0.01% to more than 90% represents a further key challenge (7, 8, 23, 46). There are clinical situations in which cfDNA concentrations are below optimal amounts for the detection of mutations. Therefore, the use of ctDNA as a diagnostic tool and for the detection of minimal residual disease requires highly specific markers and analytically sensitive techniques.

One strategy for solid tumors may be the detection of recurrent somatic rearrangements as was previously

shown for monitoring of residual disease burden in leukemia (47). McBride et al. mapped genomic rearrangements in 3 cancers and showed that PCR assays for rearrangements could detect a single copy of the tumor genome in plasma without false positives (48). It should be noted that not all rearrangements present in a tumor are indeed implicated in tumor formation or progression and that some of these passenger rearrangements might get lost in a relapsing clone or metastasis. Further recently reported diagnostic approaches involve the blood-based CRC screening test using the *SEPT9* biomarker that specifically detects a majority of CRCs of all stages and colorectal locations (49) or the use of plasma EBV DNA analysis for detecting early nasopharyngeal carcinoma in individuals without a clinical suspicion of nasopharyngeal carcinoma (50).

#### ctDNA AS A PROGNOSTIC BIOMARKER

A study from Lecomte et al. focused on *KRAS* hotspot mutations and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) hypermethylation in patients with CRC. They demonstrated that the 2-year survival rate was 100% in patients with no evidence of ctDNA who possess *KRAS* mutations or *CDKN2A* gene promoter hypermethylation, which can be found in 40% or 20%–50% of CRC patients, respectively, suggesting a prognostic value for these markers. Therefore, the presence of ctDNA in plasma seems to be a relevant prognostic marker for patients with CRC and may be used to identify patients with a high risk of recurrence (51) (Fig. 2). This was also confirmed by a study from Diehl et al., in which patients who had detectable ctDNA after surgery generally relapsed within 1 year (52). Furthermore, it was shown that high concentrations of cfDNA and mutant *KRAS* were clear indicators of a poor outcome for metastatic CRC patients (53). Similar data were reported for a set of breast cancer patients (54). Moreover, recent studies indicated that ctDNA appeared to be a better prognostic marker than CTC count when combined analysis of tumor-specific mutations in ctDNA and CTCs was performed (55). Similar results were obtained in a study with advanced non-small cell lung cancer patients, in which the analysis of ctDNA achieved a greater sensitivity for mutation detection than CTCs and detected mutations were strongly consistent with the mutation status of the matched tumor (56).

#### ctDNA AS A PREDICTIVE BIOMARKER

One of the most widespread and important applications of the liquid biopsy is the monitoring of response to therapy, in particular focusing on those therapies with known resistance mechanisms. Colorectal tumors that are wild type for *KRAS* are often sensitive to EGFR blockade, but almost all patients develop resistance within a few months (57). The liquid biopsy circumvents the bar-

rier of repeated posttreatment tumor tissue sampling and may provide a comprehensive picture within or among various tumor lesions.

A recent study suggested that circulating cfDNA provides a better overall representation of the malignant disease and could be a reliable source of diagnostic DNA, which could replace the use of tumor tissue in a diagnostic setting (58). In 2012 Diaz et al. tested whether mutant *KRAS* DNA could be detected in the circulation of CRC patients receiving monotherapy with panitumumab. Detectable mutations in *KRAS* were present in sera in 38% of patients between 5 and 6 months following treatment (59). Another study analyzing patients with metastatic CRC before application of targeted therapy showed 100% diagnostic specificity and sensitivity for the B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) V600E mutation and 98% specificity and 92% sensitivity for 7 tested *KRAS* point mutations. Interestingly, the amount of mutated alleles was highly variable (0.5%–64.1%, median 10.5%) among mutated samples (8). Several studies reported that the development of resistance to anti-EGFR therapies was associated with acquired gains of *KRAS* that occurred either as novel focal amplifications or as high-level polysomy of chromosome 12p (60, 61) and that resistant clones in the circulation were detectable months before progression was clinically obvious (60, 62). In addition, focal amplifications of other genes were recently shown to be involved in acquired resistance to anti-EGFR therapies, such as MET proto-oncogene, receptor tyrosine kinase (*MET*) and *ERBB2* (60–63). Overrepresentation of the *EGFR* gene was associated with a good initial anti-EGFR efficacy (60).

#### Methodological Aspects

Generally, there are 2 approaches for the analysis of plasma DNA (Fig. 3). The first is a targeted approach that includes the analysis of known genetic changes from the primary tumor in a small set of frequently occurring driver mutations with implications for therapy decisions, such as mutations in *KRAS* or *EGFR*. The second involves an untargeted approach without knowledge of any specific changes present in the primary tumor. Genome-wide analysis of ctDNA can be used for discovery of tumor-specific alterations in the context of disease monitoring, detection of molecular resistance, and identification of new therapeutic targets. A more cost-effective approach than whole genome sequencing is exome sequencing, which also does not require prior knowledge of the genetic landscape of the tumor.

#### TARGETED APPROACHES FOR THE ANALYSIS OF LIQUID BIOPSIES

The identification of somatic point mutations in cfDNA was published in 1994 (64). Since then, numerous studies

have analyzed known tumor-specific changes in plasma and serum. Owing to technological advances, the analytical sensitivity of detection improved dramatically in recent years and new technologies, including ARMS (amplification refractory mutation system) (53), digital PCR (dPCR) (65, 66), and beads, emulsions, amplification, and magnetics (BEAMing) (52) allow the identification of mutant alleles at very low frequencies. Newman et al. introduced cancer personalized profiling by deep sequencing (CAPP-Seq), an economical and ultrasensitive method for quantifying ctDNA (67). This method combines optimized library preparation methods for low-input DNA with a multiphase bioinformatic approach to design a “selector” consisting of biotinylated oligonucleotides that target recurrently mutated regions in the cancer of interest (67). ctDNA was detected in 100% of patients with stage II–IV non–small-cell lung carcinoma and in 50% of patients with stage I, with 96% diagnostic specificity for mutant allele fractions down to approximately 0.02% for predefined mutations. Concentrations of ctDNA were highly correlated with tumor volume and were able to distinguish between residual disease and treatment-related imaging changes. Also, measurement of ctDNA concentrations enabled an earlier assessment of response than radiographic approaches. A very recent study from Bettessowda et al. analyzed a large set of cancer patients with different tumor entities and tumor stages (7). The diagnostic sensitivity of ctDNA for detection of clinically relevant *KRAS* gene mutations was 87.2%, with a specificity of 99.2%. However, they used highly sensitive analytical methods including dPCR and the Safe-Sequencing System (Safe-SeqS) method, which was previously established by the same group (68). This approach represents an effective method to detect tumor-specific mutations even at very low levels and allows for a clear distinction from background signals by decreasing the presumptive sequencing errors by at least 70-fold. In study from Bettessowda et al. it was shown that Safe-SeqS was able to detect one mutant template in the DNA from 5 mL plasma (7). Furthermore, the authors screened for highly tumor-specific translocations that could be used to detect tumor-specific changes at very low levels or to identify minimal residual disease.

However, most of these methods interrogate only a few loci, and mutations in genes that lack mutational hotspots, such as tumor suppressors, are missed. One possibility to include driver genes without hotspots is targeted resequencing of selected genes that are known to be associated with tumorigenesis and progression. In a study from our group, we were able to identify structural rearrangements directly from plasma after targeted enrichment of chromosomal regions that are frequently involved in translocations (69). A comprehensive analysis of more than 4000 tumor samples, including over 3600 data sets from The Cancer Genome Atlas (TCGA) and

an independent non-TCGA cohort, revealed that in 76% of all occurrences from 10 tumor entities, at least 1 mutation in a panel of 25 selected genes could be identified (70). Nevertheless, the identification of mutations at low allele frequencies across sizeable genomic regions or in a few nanograms of fragmented template from cfDNA has been more challenging. To track down tumor-specific mutations in plasma, Forshew et al. developed the so-called tagged-amplicon deep sequencing (TAm-Seq) (71), including 5995 genomic bases for low-frequency mutations. Using this approach, the investigators identified cancer-specific mutations present at allele frequencies as low as 2%, with a diagnostic sensitivity and specificity of >97%. Screening of a smaller set of known mutations could achieve a detection limit of approximately 0.2%.

#### UNTARGETED APPROACHES FOR THE ANALYSIS OF LIQUID BIOPSY

The main advantage of genome-wide analysis of a liquid biopsy is that this approach is applicable to all patients because it does not rely on recurrent genetic changes. Although our group showed that genome-wide copy number profiles could be established from plasma using array-CGH (23), NGS-based approaches were able to increase the resolution of copy number analyses from cfDNA. The group of Dennis Lo was among the first to establish genome-wide profiles from plasma (11) and they further developed this technique by a combined assessment of hypomethylation and cancer-associated copy number aberrations (10). The authors showed that tumor-associated copy number aberrations (CNAs) could also be established from the bisulfite DNA sequencing data and could be used for the detection of nonmetastatic cancer cases. Plasma hypomethylation gave a diagnostic sensitivity and specificity of 74% and 94%, respectively. A reduction of the sequencing depth to 10 million reads was found to have no adverse effect on sensitivity and specificity. Our group demonstrated that CNAs can even be reliably detected with about 4 million reads generated on the benchtop sequencing MiSeq platform (Illumina) (69). Leary et al. developed a whole genome sequencing–based method called personalized analysis of rearranged ends (PARE) to identify translocations in solid tumors and applied this approach to plasma DNA samples, with which they identified several chromosomal copy number changes and rearrangements, including amplification of cancer driver genes such as *ERBB2* and cyclin-dependent kinase 6 (*CDK6*) (46). Because most cancers harbor multiple chromosomal alterations that are unlikely to be present in normal cells, this represents a highly specific approach. However, similar to a study from Thierry et al. (8) with mutant alleles ranging from 0.5% to 64.1%, the concentrations of circulating tumor DNA in this study varied dramatically, ranging



from 1.4% to 47.9% (46). Murtaza et al. performed exome sequencing of plasma DNA samples and followed multiple courses of treatment (72). Quantification of allele fractions in plasma identified increased representation of mutant alleles in association with emergence of therapy resistance. The authors concluded that exome-wide analysis of circulating tumor DNA could complement current invasive biopsy approaches to identify mutations associated with acquired drug resistance in advanced cancers (72).

Dawson et al. recently reported the use of whole genome sequencing in combination with a personalized assay to quantify circulating tumor DNA in serially collected plasma samples in metastatic breast cancer (54). After establishing patient-specific mutations using whole genome sequencing, these mutations were used to assess the concentrations of ctDNA in serial samples. The investigators observed that mutant ctDNA concentrations showed a greater dynamic range and greater correlation with changes in tumor burden than did CA15-3 (carbohydrate antigen) or circulating tumor cells. Similar to other studies, the main limitation for this approach is the requirement for prior knowledge of aberrations—whether structural or on the nucleotide level—to design a personalized assay. Another group took advantage of Affymetrix SNP 6.0 arrays for profiling of CNAs and loss of heterozygosity (LOH) in a set of 65 breast cancer patients. They identified a focal high-level DNA amplification in paired tumor and cfDNA clustered in a number of chromosome arms, some of which harbor genes with oncogenic potential. Remarkably, in the follow-up samples of 50 patients, specific CNAs that were already present in the primary tumor were detected in cfDNA, despite no other evidence of disease for up to 12 years after diagnosis (73). This indicates that a noninvasive approach might be used to detect dormancy/minimal residual disease in patients during follow-up.

One drawback of genome-wide methods is the lack of analytical sensitivity and, despite strong efforts to lower the error rate of MPS to improve detection limits, which is exemplified by the recently proposed duplex method (74), none of these methods has been applied yet for the comprehensive analysis of tumor genomes from plasma (Fig. 3). Nevertheless, there are numerous approaches to analyze tumors noninvasively, but it is not yet clear which method will emerge as the best one. Methods based on low-coverage sequencing for copy number analysis can on the one hand provide clinically relevant information in a cost-effective and fast manner (10, 60, 69). On the other hand, these approaches lack analytical sensitivity and cannot detect mutations on the nucleotide level. At the moment, however, increasing depth still means increased costs and time, which is an obstacle for actual clinical implementation. Considering the recent drop of sequencing costs from 108.065 USD per genome

in 2009 to 4.920 USD in 2014 [these figures account for labor, administration, management, utilities, reagents, and consumables (<http://www.genome.gov/sequencing-costs/>)] and improvement of speed, whole genome sequencing at an appropriate depth of cfDNA will eventually be readily available for clinical purposes. Moreover, newer sequencing technologies such as nanopore sequencing are starting to emerge and might provide improved accuracy and reliability without the need for amplification, thus improving sensitivity by removing a major source of bias.

## Clinical Implementation of Liquid Biopsy

To date there are only few large controlled studies available in which the analysis of ctDNA was implemented in clinical trials. One study including 105 patients with solid tumors, who were referred for participation in phase I trials of molecularly targeted drugs using the Sequenom MassArray System and OncoCarta panel for somatic mutation profiling, demonstrated that a liquid biopsy was beneficial in advanced cancer patients when repeated tumor biopsy was not safely feasible and genomic analysis of archival tumor was deemed insufficient (75). Schwarzenbach et al. conducted a multicenter study that recruited a cohort of 388 patients with primary breast cancer before chemotherapy and investigated LOH in a set of 8 genes (76). Another study with 108 metastatic CRC patients monitored the abundance of mutant *KRAS*/*BRAF* alleles in plasma at baseline and before each cycle of third-line treatment with cetuximab and irinotecan (77). cfDNA and *KRAS* concentrations decreased from baseline to cycle 3 and increased at the time of progression, and loss of mutations was associated with a benefit of treatment, whereas the appearance of mutations during therapy may have been responsible for acquired resistance in primary wild-type disease (77). The same group investigated total cfDNA in CRC patients during treatment with second-line chemotherapy and cfDNA in healthy controls as well as patients with different comorbidities (78). cfDNA concentrations were significantly higher in CRC patients than in controls, and patients with high concentrations had a shorter survival from irinotecan than did patients with lower concentrations (78). Also, the combination of marker analysis with plasma *KRAS* mutations added further prognostic impact (78).

The potential of the liquid biopsy in the field of clinical cancer research is being clearly recognized and a liquid biopsy is now frequently embedded in the design of several clinical trials. However, for actual implementation of the liquid biopsy in clinical practice, it is necessary to develop standardized preanalytical and analytical methodologies, including blood collection, processing, and storage and DNA extraction, quantification, and validation in large prospective clinical studies. Logistical

sampling is facilitated only in the course of controlled studies, thereby providing pretreatment and follow-up samples in a statistically powerful sample size. Progress in the clinical implementation can be achieved only if long-term studies with adequate sample sizes are performed and results obtained from such studies are correlated with disease-free survival/overall survival and other clinical settings. Furthermore, because the dynamics of the release of ctDNA have not been fully elucidated and, therefore, the timing of ctDNA analysis in relation to therapy may be important, multiple samples at different time points should be obtained from the same patients. For instance, monitoring ctDNA shortly after and during drug administration might reveal various allelic fractions of ctDNA, and may provide valuable information on kinetics of ctDNA release.

### Future Perspectives

Numerous data have shown that the analysis of ctDNA provides complementary tools in the diagnosis, prognosis, and management of cancer patients; therefore ctDNA can be used as a noninvasive cancer biomarker. Although biomarker discovery is generally a growing field, many tumor entities lack recurrent genetic changes, highlighting the need for uncovering specific cancer signatures and improved analytical and diagnostic sensitivity of genome-wide analyses. Moreover, there is a need for a greater focus on the complexity of intratumoral heterogeneity. It is not clear yet whether ctDNA is representative of all relevant metastatic cell clones located at different sites or whether ctDNA represents DNA from distinct subclones that can promote clinical progression and/or therapeutic resistance. Therefore, further clinical evaluations, comparative sequence analyses of plasma DNA, and biopsies in combination with imaging studies and detailed functional studies are needed to assess clinical progression and/or therapeutic resistance in more detail. Recent studies have shown that we certainly cannot assume that intratumoral heterogeneity is solely based on genetic changes (79); therefore, epigenetic changes also have to be taken into account, thereby adding another level of complexity. Epigenetic changes in general are considered as an early event in carcinogenesis

and might therefore be a suitable marker for early detection. Furthermore, epigenetic targets established noninvasively are emerging as effective and valuable approaches to chemotherapy as well as chemoprevention of cancer.

Taken together, despite all efforts to develop suitable tools for a comprehensive analysis of tumor genomes from plasma DNA, most laboratory processes are currently too time-intensive and costly for actual implementation in a diagnostic setting. However, sequencing costs will drop further and this field of technology is continuously evolving. As a result, it is just a matter of time before technical advances and cost reductions may allow for the implementation of genome-wide approaches with high resolution to be used as a routine tool in laboratory medicine. The analytical sensitivity of methodologies is furthermore dictated by the dramatic variability in the abundance of mutated fragments in the circulation. This is particularly a crucial factor in settings in which the amount of tumor-specific DNA is too low for currently available techniques, i.e., early stages or minimal residual disease. All currently used NGS-based methods that depend on amplification of sensitivity are limited by the error rate of DNA polymerases, which is generally considered to be 0.01%. However, emergence of third generation sequencing methods will minimize issues related to the biases introduced by PCR amplification and dephasing.

Irrespective of technical limitations, future developments will need to provide clinical standards to validate the liquid biopsy as a clinical biomarker in well-designed and sufficiently powered multicenter studies.

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