

EXPERT
REVIEWS

Diagnostic and prognostic value of circulating tumor-related DNA in cancer patients

Expert Rev. Mol. Diagn. 13(8), 827–844 (2013)

Diego M Marzese[‡],
Hajime Hirose[‡] and
Dave S B Hoon^{*}

Department of Molecular Oncology,
John Wayne Cancer Institute,
2200 Santa Monica Blvd, Santa
Monica, CA, 90404, USA

^{*}Author for correspondence:
hoon@jwci.org

[‡]Authors contributed equally

Qualitative and quantitative analysis of circulating cell-free DNA (cfDNA) is an emerging non-invasive blood biomarker utilized to assess tumor progression and to evaluate prognosis, diagnosis and response to treatment. There is a need to develop cfDNA biomarkers to avoid complex risk-prone biopsy procedures for primary or metastatic tumors. Given the challenges associated with inter- and intra-tumor heterogeneity, the implementation of genome-wide cfDNA analysis will become an important avenue to understand tumor progression and therapeutic settings, not only for predominant, but also for under-represented tumor subclones with specific genomic aberrations. We summarize the latest publications in cfDNA analysis, including a metric analysis of clinical trials and new high-throughput technology applied to cfDNA analysis in clinical oncology.

KEYWORDS: biomarkers • cancer • circulating cell-free DNA • diagnostic • epigenomics • genomics • metastasis • mutation • prognostic • serum and plasma DNA

Circulating cell-free DNA (cfDNA) has become increasingly popular for its use as a less-invasive diagnostic and prognostic liquid biopsy for cancer patients. The approach provides broad and descriptive genomic and epigenomic information that does not require direct tumor assessment. The potential clinical utility of this blood biomarker is based on quantitative and qualitative characteristics of tumor-associated cfDNA detected in the peripheral blood of cancer patients. Thus, tumor-related genetic and epigenetic alterations, as well as the overall integrity of cfDNA in plasma or serum, have been used to distinguish cancer patients from healthy counterparts, and to monitor treatment efficiency and disease progression. Circulating tumor cell (CTC) analysis represents another source of information from the liquid biopsy that has now been applied to different cancer types [1–4]. The most relevant characteristics of cfDNA and CTC approaches, in terms of advantages, complementarity and provided information, have been the focus of previous articles [5–7].

Recently, major high-throughput technological advances in cfDNA analysis have been achieved, which has moved the field of blood-based monitoring to a higher level of detail and information. This review is focused on the diagnostic and prognostic values of cfDNA in cancer patients, giving priority to reports published within the past 5 years involving cohorts of at least 50 patients, referencing of previous landmark publications and presenting the latest advances in next-generation sequencing (NGS) analysis. For a more extended view of the historic background, origin of cfDNA and clinical studies, we recommend previous comprehensive reviews in this field [5,6,8–10].

Clinical implications of cfDNA assessment

Currently, treatment of solid tumors requires multimodal management. Choosing the most appropriate and effective treatment modalities is a significant decision toward the eradication or control of cancer. Genomic biomarkers have become useful for drug targeting as well

as diagnosis. However, the availability of tissue to assess represents a problem. There is an increasing need for the evaluation and identification of prognostic and diagnostic genomic biomarkers through biopsy, imaging and blood-based tests. As new targeted-drugs are combined with radiotherapy and surgery, the assessment of patient response becomes more complex and requires better biomarker surrogates. This has become a major issue for oncology whereby US FDA approval of efficient blood cancer biomarkers has significantly lagged behind.

To date, tumor biopsy remains the most reliable technique to establish a correct diagnosis. However, the accessibility of tumors in some organs such as pancreas or brain is limited and biopsy of them can expose the patient to injury, tumor spread and/or inflammation. It has been reported that biopsies of some solid tumors have caused recurrence due to needle-track seeding; thus, this approach involves a high-risk of disease spread [11]. Also, small tumors (<1.5 cm) represent a technical challenge for detection and a limitation for biopsy. During the past several years, imaging has shown a significant improvement for cancer diagnosis. However, these techniques pose crucial resolution limitations, which results in a minimum lesion size requirement, along with difficulty in distinguishing well-differentiated malignant tumors from benign tumors [12,13]. The use of approved blood-based biomarkers, including CEA, CA19-9, CA15-3 and PSA, among others, allows for diagnosis and follow-up of different tumor types. Nevertheless, these biomarkers do not always show good concordance with clinical tumor status, and in some cases, are poorly discriminative between cancer and normal tissue or benign disease. Even when combined, biopsy, imaging and blood biomarkers are often not well co-ordinated to identify cancer progression. Because more treatment options are available, there is a greater need to evaluate tumor status more frequently than before, to aid in rapid decision making to increase treatment efficacy.

cfDNA has the potential to become a more efficient blood-based biomarker that can accurately provide information about the molecular alterations present in the tumor, as well as monitor tumor clones with specific genomic signatures. This is especially important for patients with inaccessible primary tumors or multiple metastases to distal organ sites. cfDNA studies have been undertaken for several decades; however, the field recently has gained more interest with the development of new high-throughput technological approaches. Genome testing of fetal cfDNA has rapidly progressed, and after large patient cohort validation by many different groups, the assay is now routinely used in clinical settings [14,15]. In oncology, NGS has allowed the identification of novel targetable genomic aberrations in different solid tumors. These new genomic targets are now becoming the focus of intense research in cfDNA, leading to a more personalized and efficient cancer treatment. Although the assays are early in development, the limitations to be addressed include cost, accuracy, sensitivity and clinical utility validation. These issues will be discussed later in the review.

Origin of circulating cfDNA

Nucleic acids can be released by various pathological and normal physiological mechanisms, such as active or passive release from living or dying cells. cfDNA can be found in a variety of extracellular media including the blood, cerebral spinal fluid, sputum, stool and urine, among others [8,10]. In the blood circulation cfDNA is detected in different forms, such as free-floating DNA, protein-bound DNA (nucleosomes and vitrosomes) and vesicle-bound DNA (apoptotic bodies and microvesicles) (FIGURE 1) [10,16,17].

It has been suggested that dead or physically damaged cells represent the predominant source of cfDNA in the blood [18]. The apoptotic process generates DNA fragments of approximately 180 bp in length as a consequence of a programmed digestion of the genomic DNA (FIGURE 1) [19,20]. In solid tumors, cfDNA can be also released through necrosis, autophagy, necroptosis and other physiological events induced by microenvironmental stress and chemotherapy treatment [21–23]. Unlike apoptosis, necrosis is considered as a pathological process, which generates larger DNA fragments due to an incomplete (and random) digestion of genomic DNA (FIGURE 1) [24]. Importantly, the length (or integrity) of cfDNA can potentially distinguish between apoptotic and necrotic origin, which will be described further in the following section.

Nevertheless, not all cfDNA is from cell death origin. *In vitro* experiments using cultured lymphocytes have shown that, regardless of cell death mechanism, living cells spontaneously release newly synthesized DNA as part of a homeostatically regulated system [9,25,26]. Other studies have also shown that the stimulation of lymphocytes (with tuberculin or phytohemagglutinin) leads to the release of large amounts of DNA in absence of cell death processes [9,27,28].

Solid tumors represent a complex mixture of different normal cell types and cancer subclones with different hierarchy and differentiation stages. Therefore, it is important to accurately assess the amount and alteration of cfDNA, which can reflect the heterogeneity, tumor burden and doubling time of multiple cancer and normal cells.

Quantitative analysis of cfDNA

The initial landmark quantitative assessment of cfDNA in cancer was published by Leon's group [29]. They observed that patients with metastatic disease presented higher cfDNA levels, which decreased as patients improved in clinical status after radiation therapy. After this seminal publication, different studies focused on quantifying approaches to ratify the higher level of cfDNA in different cancer patients compared with healthy individuals [30,31]. Sozzi *et al.* identified elevated plasma cfDNA (mean = 381 ng/ml) in patients with non-small-cell lung carcinoma (NSCLC) compared with control individuals (mean = 18 ng/ml). In this study, the concentration of cfDNA, in a subgroup of relapse-free individuals, was significantly lower (mean = 34 ng/ml) than in a subgroup of patients presenting recurrence of the disease (mean = 375 ng/ml) [32]. In a study involving 175 breast cancer patients and 80 healthy controls,

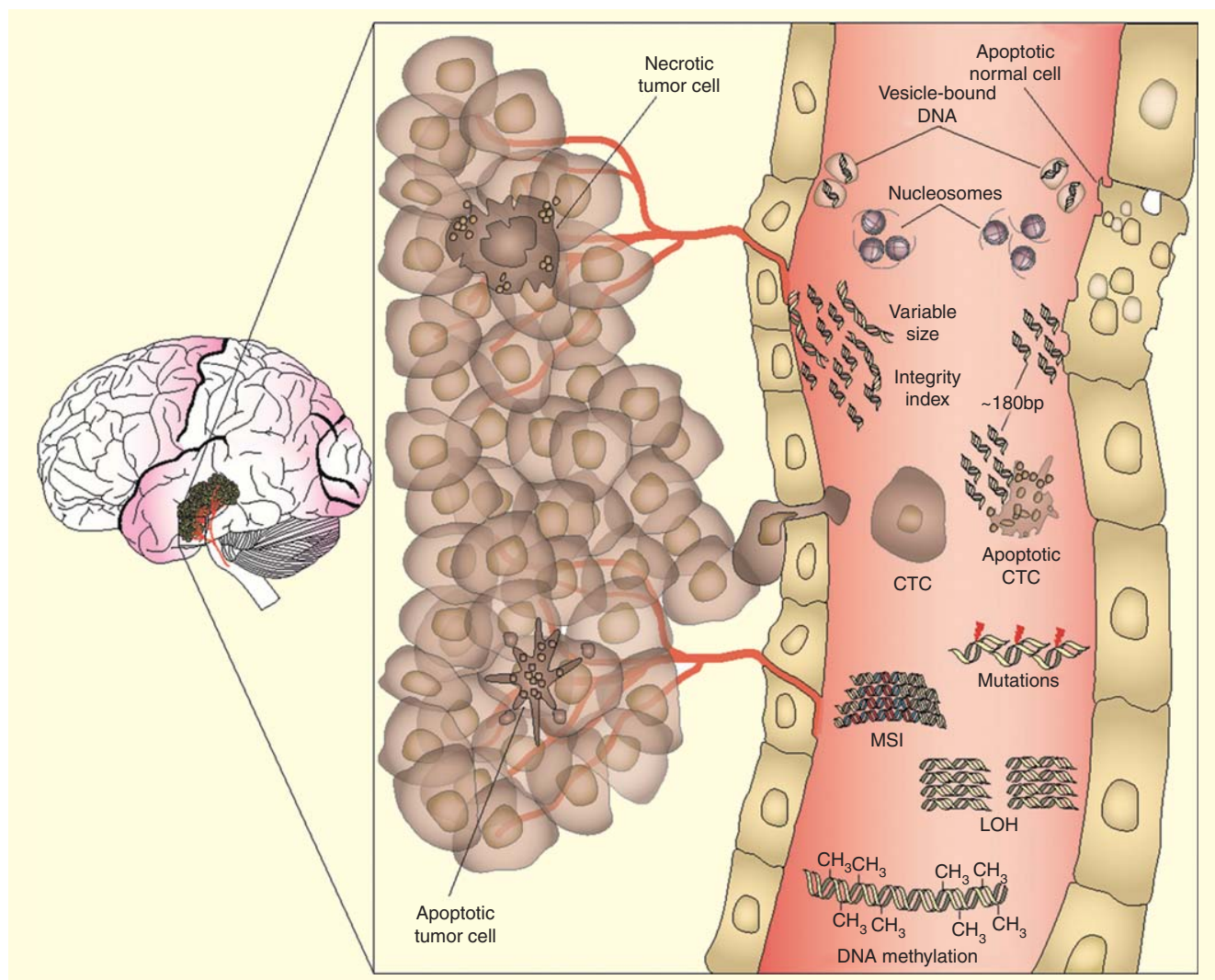


Figure 1. cfDNA origin and tumor-associated alterations present in circulation plausible to be detected by molecular analysis.

the diagnostic potential of cfDNA quantification was described [33]. The increase of cfDNA levels according to cancer progression was also recently reported [34]. In this study, the author identified increasing levels of cfDNA in correlation with tumor size, lymph node involvement, higher grade and advance stages in breast cancer patients. The utility of total cfDNA levels has been used as a factor in recurrence prediction of different cancer types (TABLE 1).

The classical methods applied to estimate the amount of cfDNA involve quantitative real-time PCR-based (qPCR), fluorescence-based (including Hoechst dye or PicoGreen staining) and spectrophotometric-based (including ultraviolet spectrometry) approaches [35,36].

Fluid specimen collection, processing, storage and DNA extraction represent key technical factors that affect specificity and sensitivity of the quantitative analysis [8]. Gormally *et al.* reported that the quantification of cfDNA in plasma of 776 healthy controls was highly variable across 23 centers [37].

The results indicate that differences in processing protocol are accountable for this variation. Generally, serum has higher levels of cfDNA than plasma [38]. However, it has been controversial whether serum would yield more false positives due to cfDNA from normal cells. Our serum cfDNA analysis, involving filtration prior to cryopreservation, has been shown to yield quality cfDNA for various genomic assays [39]. It is important to note, however, that serum does contain non-tumor specific cfDNA. Then the issue becomes how specific the assay is.

Quantification of cfDNA may be an effective tool for detection of tumor burden as well as a prognostic indicator. However, an increased cfDNA level is also seen in non-cancerous normal and pathological processes, such as inflammation [40], pregnancy [41], preeclampsia [42], trauma [43], myocardial infarction [44], exercise [45] and aging [46]. Therefore specificity of the cfDNA biomarker becomes highly important. Furthermore, a recent study reported by Jylhävä *et al.* [47], including 1841 young healthy individuals, showed that the level of serum

Table 1. cfDNA quantification assessment.

Cancer type	Diagnostic	Prognostic	Ref.
Breast	Catarino <i>et al.</i> (2008) Roth <i>et al.</i> (2011) Hashad <i>et al.</i> (2012) Gong <i>et al.</i> (2012) Perkins <i>et al.</i> (2012)	Divella <i>et al.</i> (2009) Hashad <i>et al.</i> (2012) Perkins <i>et al.</i> (2012)	[33,116] [113,34] [34,115] [114] [115]
Brain	Shi <i>et al.</i> (2012)		[117]
Colorectal	Schwarzenbach <i>et al.</i> (2008) Danese <i>et al.</i> (2010) Perkins <i>et al.</i> (2012)	Perkins <i>et al.</i> (2012)	[118,115] [119] [115]
Gastrointestinal	Park <i>et al.</i> (2009) Tomochika <i>et al.</i> (2010) Park <i>et al.</i> (2012)		[120] [121] [122]
HCC	Chen <i>et al.</i> (2012)		[123]
Melanoma	Pinzani <i>et al.</i> (2011) Salvianti <i>et al.</i> (2012) Perkins <i>et al.</i> (2012)	Perkins <i>et al.</i> (2012)	[61,115] [111] [115]
Ovarian	Zachariah <i>et al.</i> (2008) Perkins <i>et al.</i> (2012)	Kamat <i>et al.</i> (2010) Perkins <i>et al.</i> (2012)	[124,125] [115]
Lung	Paci <i>et al.</i> (2009) Ulivi <i>et al.</i> (2013) Yoon <i>et al.</i> (2009)	Ulivi <i>et al.</i> (2013)	[126,127] [127] [128]
Prostate	Schwarzenbach <i>et al.</i> (2009) Perkins <i>et al.</i> (2012)	Perkins <i>et al.</i> (2012)	[129,115] [115]

cfDNA is associated with at least 110 single-nucleotide polymorphisms, all of which are located in the UGT1 locus, which catalyzes detoxification of several drugs. Together, this evidence highlights the essential need to identify tumor-specific cfDNA features to avoid the influence of cfDNA released by non-cancerous physiological events.

Qualitative analysis of cfDNA

cfDNA bearing tumor-related genomic and epigenomic alterations represent a unique source of information about the respective patient's tumor(s). Assessable cfDNA genomic alterations include the degree of integrity, mutations (mt), rearranged genomic sequences, copy number variations (CNV), microsatellite instability (MSI), amplified sequences, loss of heterozygosity (LOH) and DNA methylation (DNAm).

Evaluation of cfDNA integrity

A different approach to identify tumor-related cfDNA alteration is to evaluate the cfDNA integrity index, which is independent of any specific genomic change such as mt or translocations. Individual cancer types have a generally low frequency of tumor-related mt with exceptions such as *BRAF* (V600E) in melanoma, and *KRAS* (codon 12 and 13) in colorectal, pancreas and NSCLCs [48–50]. This represents a problem in the diagnosis of disease, because no single biomarker is universal among cancers. Jahr *et al.* proposed that the distribution of DNA fragment size could be used to determine the origin

of cfDNA from either apoptotic or necrotic cells [51]. As previously mentioned, programmed cell death under normal conditions is accompanied by an orchestrated physiological degradation of the genomic DNA, which results in small and uniform DNA fragments (~180 bp). In contrast, cell necrosis, which is a frequent event in solid tumors, generates a wide spectrum of DNA fragment lengths as a consequence of random digestion (FIGURE 1).

The DNA integrity index has been assessed by qPCR using different target such as coding genes or non-coding repeated genomic sequences. Increased DNA integrity was initially reported in the plasma of gynecologic and breast cancer patients [24]. The integrity index was derived from the threshold cycle number assessed by qPCR for 2 amplicons (one of 400 bp and the other of 100 bp). Umetani *et al.* developed a method using qPCR to directly measure the integrity of cfDNA without the need of DNA purification [52]. In this case, the integrity index was calculated as the ratio between a 115-bp amplicon (amplification of apoptotic fragments) and a 247-bp amplicon (amplification of necrotic fragments) of the non-coding repeated sequences *ALU*. *ALU* sequences are non-intandem repeat sequences of approximately 300 bp long that are found on all chromosomes, representing the most abundant transposable elements of the human genome. *ALU* elements are classified as short-interspersed elements. In addition, our group has also shown the utility of long-interspersed nucleotide element-1 (*LINE1*), a 6–8 kb long CpG-poor DNA sequence,

Table 2. cfDNA integrity index.

Cancer type	Diagnostic	Prognostic	Ref.
Bladder	Hauser <i>et al.</i> (2012) Ellinger <i>et al.</i> (2012)	Ellinger <i>et al.</i> (2008)	[130,63] [131]
Breast		Umetani <i>et al.</i> (2006)	[54]
Brain	Shi <i>et al.</i> (2012)		[117]
Colorectal	Mead <i>et al.</i> (2011) Umetani <i>et al.</i> (2006)		[132] [52]
HCC		Chen <i>et al.</i> (2012)	[123]
Melanoma	Pinzani <i>et al.</i> (2011) Salvianti <i>et al.</i> (2012)		[61] [111]
Ovarian	Salani <i>et al.</i> (2007)		[133]
Prostate	Ellinger <i>et al.</i> (2008)	Ellinger <i>et al.</i> (2008)	[64]
Renal	Gang <i>et al.</i> (2010)		[59]

which represents almost 17% of the human genome, to evaluate cfDNA integrity in breast cancer patients [53]. cfDNA integrity index showed clinical relevance by discriminating between lymph node negative and positive breast cancer patients [54]. The persistence of high DNA integrity index after radiotherapy has also been associated with poor prognosis of nasopharyngeal carcinoma patients and with poor response to pre-operative chemotherapy in rectal carcinoma patients [55,56].

Higher cfDNA integrity in cancer patients compared with the healthy counterpart has also been demonstrated in multiple cancers such as breast, colorectal, esophagus, head and neck, kidney, melanoma, nasopharyngeal, perianthelial and prostate cancer [52,54,57–62]. The latest key and landmark reports in this field are presented in TABLE 2.

Contrary to the identification of high cfDNA integrity index, other studies have reported decreased DNA integrity in cancer patients compared with the healthy controls [63–65]. In future studies, DNA integrity assays need to be better designed and adapted to the newer technologies. Performing NGS of non-coding genomic regions (such as *ALU* and *LINE1*) will likely expand the application of this complex and promising field.

Detection of tumor-specific mutations & copy number variations in cfDNA

Identification of cfDNA mt concordant to the tumor mutational spectrum provides strong evidence that the detected cfDNA is released from the primary or metastatic tumors. In 1994, two seminal publications described *KRAS* and *NRAS* mt in the blood of pancreatic carcinoma and leukemia patients, respectively [66,67]. During the past decade, an extensive body of mt has been used to detect cfDNA in the blood of cancer patients (TABLE 3).

Dawson *et al.* recently reported the use of whole-genome and targeted sequencing to assess the landscape of genetic mt in the primary tumors of 30 breast cancer patients and consequently, they designed a personalized assay to screen for genetic alterations in ctDNA [7]. They observed that response to treatment

and prognosis of the patient negatively correlated with the amount of cfDNA. However, even when using whole-genome sequencing (WGS), no single locus alteration (mt or structural variations (SNV)) was found to be common among all the patients. Combining the most frequently mutated genes (*TP53* and *PIK3CA*) with other SNV, tumor-associated cfDNA was successfully detected in 29 (out of 30) patients. Unfortunately, these mt are currently not actionable targets for treatment.

The major limitation of this approach is the requirement for prior knowledge of the tumor mt profile to then design a cfDNA detection assay. Genetic mt of the primary tumor (or metastasis) are usually evaluated from a limited segment of the whole tumor mass obtained from biopsy or tumor surgically resected. New evidence points toward a significant heterogeneity in the DNA copy number and mt profile between primary and metastasis, or even within a single tumor mass [68–72]. A common issue of this profound heterogeneity is that it has been indicated that drug resistance in solid tumors may be pre-determined by the existence of subclones under-represented or not detected due to assay sensitivity in the initial biopsy employed to decide the therapeutic protocol [73,74]. Unfortunately WGS of tumor sample may not represent the entire tumor mass being assessed. It is obvious that minor clones of genomic aberrations may not be accurately detected. As we have now seen, sequencing of tumors may lead to biased interpretation of genomic aberration events as tumors can be heterogeneous so that sampling is a key issue.

Genomic tumor mutation profile assessed by next generation sequencing of cfDNA

To generate a non-invasive, unbiased tumor-associated mt profile, the direct application of NGS to cfDNA is a great alternative. In 2013, Chan *et al.* published the application of massively parallel sequencing to characterize SNV in cfDNA of four hepatocellular carcinoma patients. To identify tumor-associated CNV, DNA was extracted from paired tumor and

Table 3. cfDNA mutation and copy number analysis.

Cancer type	Diagnostic	Prognostic	Ref.
Breast	Page <i>et al.</i> (2011) Perkins <i>et al.</i> (2012) †Leary <i>et al.</i> (2012) †Heitzer <i>et al.</i> (2013) †Dawson <i>et al.</i> (2013) †Murtaza <i>et al.</i> (2013)	Higgins <i>et al.</i> (2012)	[134,136] [115] [79] [135] [7] [76]
Cervical	Dobrzycka <i>et al.</i> (2010)	Dobrzycka <i>et al.</i> (2010)	[137]
Colorectal	Perkins <i>et al.</i> (2012) Spindler <i>et al.</i> (2012) †Leary <i>et al.</i> (2012) †Heitzer <i>et al.</i> (2013) †Murtaza <i>et al.</i> (2013)		[115] [138] [79] [135] [76]
HCC	Igetei <i>et al.</i> (2008) †Chan <i>et al.</i> (2013)		[139] [75]
Lung	Rosell <i>et al.</i> (2009) Akca <i>et al.</i> (2013) †Murtaza <i>et al.</i> (2013)	Wang <i>et al.</i> (2010) Jian <i>et al.</i> (2010) Goto <i>et al.</i> (2012) Nygaard <i>et al.</i> (2013)	[140,142] [141,143] [76,144] [145]
Melanoma	Shinozaki <i>et al.</i> (2007) Pinzani <i>et al.</i> (2010) Perkins <i>et al.</i> (2012)	Shinozaki <i>et al.</i> (2007) Board <i>et al.</i> (2009)	[146] [61,147] [115]
Ovarian	Perkins <i>et al.</i> (2012) †Forshew <i>et al.</i> (2012)	Dobrzycka <i>et al.</i> (2011)	[115,149] [148]
Prostate	Perkins <i>et al.</i> (2012) †Heitzer <i>et al.</i> (2013)		[115] [150]

†Article involving genome-wide or whole-genome analysis.

plasma samples obtained one week before and after surgical resection [75]. Concordant genome-wide SNV between tumor tissues and pre-surgical cfDNA were identified. Most importantly, the study demonstrated that the structural alterations detected in cfDNA almost entirely disappeared after surgery. This study also addresses the turnover of tumor-associated cfDNA in cancer patients. Another auspicious finding in the same report was that the CNV profile detected in the cfDNA of a patient with synchronous breast and bilateral ovarian primaries was a mixture of the three primary tumors. These authors performed the analytical dissection of the degree of contribution of each primary tumor and identified that the fractional concentration of tumor DNA in plasma and the type of CNV (1-copy losses, 1-copy gains and 2-copy gains) strongly influenced detectability in cfDNA. In this respect, large tumors are predominantly represented in the plasma-derived CNV profile, suggesting that this approach would also allow tumor load to be estimated. Murtaza *et al.* reported the whole-exome sequencing of serial plasma samples (2–5 per patient over the course of 2 years) to track the evolution of six metastatic cancers (breast, lung and ovarian) in response to therapy [76]. This approach allowed them to identify mt associated with acquired drug resistance. Their quantification of allele fractions in cfDNA described an increment of the mutant alleles according to the emergence of therapy resistance.

Mutation screening of the entire genome promises to become the ultimate, most comprehensive tool for the analysis of cfDNA. However, cost and sensitivity will continue to be an issue for its broad application. Data generated from WGS of fetal cfDNA has provided the seminal evidence to support this diagnostic process [77,78]. The most important difference for the oncology field is that the mt frequency of specific cancer types are limited and there will be a high rate of false positive without a wide screening of multiple mt. This is a concern that needs to be addressed in order for the tests to be more clinically useful.

Leary *et al.* published the first WGS analysis of cfDNA extracted from cancer patients [79]. The patient selection criteria included 10 healthy individuals and 10 late stage (3 breast and 7 colorectal) cancer patients. It is remarkable that after applying a strict statistical criterion (Bonferroni correction for multiple comparisons), SNV and CNV were identified in all cancer patients, but not in healthy subjects. This approach succeeded in identifying tumor cfDNA in concentrations of less than 1% with a sensitivity >90% and specificity >99%. Because this study included only advanced stage cancer patients, in our opinion, it will be highly important to identify how early in cancer progression this approach will be able to efficiently discriminate between cancer patients and normal individuals. Also, the number of non-cancer subjects should be increased to characterize age group, gender and non-cancer pathological

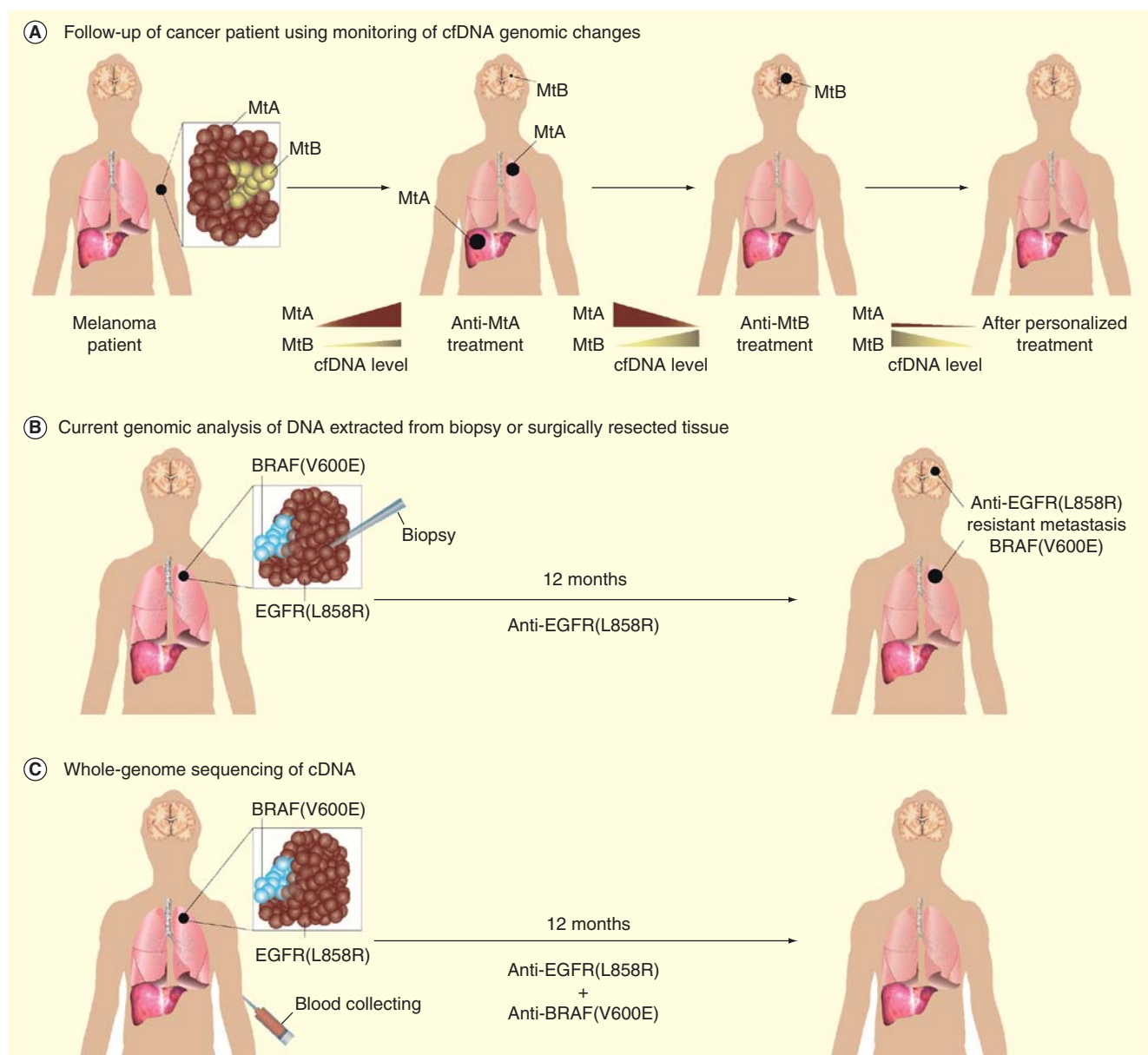


Figure 2. Potential advantages and applications of cfDNA analysis. (A) Application of cfDNA for patient treatment follow-up. In this example, a melanoma patient presents a heterogeneous primary tumor constituted by mutation profile A (MtA) and mutation profile B (MtB). The cfDNA assessment indicates a higher level of MtA as a consequence of being the predominant tumor subclone. After starting with the treatment, anti-MtA clones level in cfDNA decreased while MtB is starting to be detected and specific therapy is selected. (B) Current treatment decision based on genetic or genomic analysis of biopsies or sections obtained from surgically resected tumor tissues. In this example, the resected tumor of a patient with non-small cell lung carcinoma is analyzed revealing targetable mutations in *EGFR* gene. After one year of treatment, the patient relapses with a tumor harboring *BRAF* (V600E) mutation. (C) The application of WGS of cfDNA can identify the genetic mutation of the predominant clones (*EGFR* (L858R)) and the under-represented subclones harboring *BRAF* (V600E). In this diagnostic setting, both targetable mutations could be considered for treatment selection.

status in which the WGS will continue to show no SNV or CNV in cfDNA. Defined multicenter clinical trials with adequate sample size will be needed to address these issues. The most remarkable advantage of this approach is the independence of the knowledge about primary or metastatic tumors mt profiles. Also, it can potentially identify subclones under-represented in tissue biopsy that allows for a more personalized

and comprehensive treatment. An example of this has been observed in the discrepancies detected in the evaluation of HER2 in primary breast tumor and CTC. Patients with HER2- primary tumors and HER2+ CTC treated with trastuzumab have shown clinical response [80,81]. Using cfDNA WGS to identify under-represented tumor subclones, such as colorectal carcinomas harboring *ERBB2*, *BRAF* or *NRAS* mt, could

Table 4. cfDNA MSI and LOH analysis.

Cancer type	Diagnostic	Prognostic	Ref.
Breast	Shaw <i>et al.</i> (2012)	Schwarzenbach <i>et al.</i> (2009) Schwarzenbach <i>et al.</i> (2012)	[151,152] [90]
Brain	Lavon <i>et al.</i> (2010)		[153]
Colorectal	Schwarzenbach <i>et al.</i> (2010)		[154]
Ovarian		Kuhlmann <i>et al.</i> (2012)	[155]
Prostate	Schwarzenbach <i>et al.</i> (2008) Schwarzenbach <i>et al.</i> (2009) Sunami <i>et al.</i> (2009)		[156] [129] [110]

guide the clinical therapeutic decision. FIGURE 2 shows a representation of the potential advantages of adding cfDNA WGS to current biopsy practice in treatment decision making.

Microsatellite instability & loss of heterozygosity detected in cfDNA

MSI and LOH detection in cfDNA were first reported in 1996 by Nawroz *et al.* [82]. The authors showed a concordance between MSI and LOH alteration in cfDNA from head and neck cancer patients. During the past several years, many similar strategies have been applied to detect multiple MSI markers in different cancer types (TABLE 4). However, many of these studies have suggested a low concordance between MSI detected in cfDNA and in tumor DNA. As has been demonstrated, mismatch repair systems can fail in dying normal cells, which can therefore release cfDNA harboring MSI in normal conditions, leading to a misinterpretation of the MSI occurring in tumors [83]. Our studies in MSI and LOH have shown clinical relevance for these markers as well as utility for early stage detection of breast carcinoma, colorectal cancer and cutaneous melanoma [84–89].

Recently, a study involving a large number of breast cancer patients (n = 388) has demonstrated improvement in the identification of LOH [90]. The strategy involves an initial fractionation of plasma cfDNA into low- and high-molecular weight followed by analysis of eight polymorphic markers. This approach identified the association of LOH of *CCND2* with shorter overall survival. The initial fractionation of cfDNA can also improve the detection of MSI in cfDNA and might also provide clinically relevant information on the heterogeneity of different cancers.

Detection of tumor-associated DNA methylation in cfDNA

DNAm is a covalent chemical modification that has a pivotal role in gene expression regulation and genome stability. Methylation of cytosine residues occurs throughout the entire genome, and is often found in a sequence context of CpG. Low CpG density regions found in repetitive elements such as *LINE1*, are generally methylated in normal cells, while most of the high CpG density regions (CpG islands), especially in a gene promoter environment, are usually unmethylated [91]. A large number of studies has shown that DNAm aberrations of gene promoters, as well as non-coding genomic sequences,

are associated with tumor initiation, dissemination and metastasis establishment and progression [92]. In cancer, aberrations in genomic DNAm patterns include two converse phenomena: genome-wide demethylation focused on low CpG density regions, and gene-specific *de novo* methylation. Importantly, because of the covalent nature of the methyl (-CH₃) group bound, the DNAm modification is stable even in circulation and can be assessed to monitor tumor-related processes. In 1999, detection of aberrant DNAm in plasma and serum of patients with NSCLC, breast carcinoma and liver carcinoma was reported [93–95]. The prognostic significance of serum and plasma cfDNA methylation (cfDNAm) was later demonstrated in esophageal, colorectal and breast carcinomas [96,97].

In general, the methods available for cfDNAm assessment have been previously discussed in detail by several reviews [98,99]. cfDNAm detection methods can be classified according to the size of the analyzed target region in candidate locus (or loci), genome-wide and whole-genome cfDNAm analysis. Several studies have indicated the potential of cfDNAm as a diagnostic and prognostic marker for cancer patients (TABLE 5). The candidate locus (or loci) approach has been the strategy most employed. As we discussed in the cfDNA mt screening section, the candidate locus (or loci) approach requires prior knowledge of the tumor profile.

Recent publications suggest that the classical approach for DNAm screening, using candidate genes or only limited to CpG islands, is no longer efficient for cancer research; CpG shore and CpG shelf have demonstrated to be important for gene expression regulation [100,101]. The application of high-throughput DNAm analysis technology has demonstrated that the most relevant differentially methylated regions, between tumor and normal tissues and even between different tumor types, are predominantly located in non-CpG islands of the human epigenome [100,102]. Our group previously demonstrated in solid tumors that the DNAm analysis of non-coding regions of the cancer epigenome, such as *LINE1* or *MINT*, is highly relevant, not only for cancer diagnosis, but also for cancer prognosis [103–107]. We have also demonstrated the association of *LINE1* hypomethylation in serum cfDNA with melanoma progression and patient outcome [103].

Recently, a study demonstrated genome-wide cfDNAm profiling of esophageal cancer (tissue and matched cfDNA) in a limited number of patients (n = 8) [108]. A high concordance

Table 5. cfDNA methylation analysis.

Cancer type	Diagnostic	Prognostic	Ref.
Breast	Van der Auwera <i>et al.</i> (2009)	Sharma <i>et al.</i> (2010)	[157,158]
	Sharma <i>et al.</i> (2010)	Mirza <i>et al.</i> (2010)	[158,167]
	Sharma <i>et al.</i> (2010)	Matuschek <i>et al.</i> (2010)	[159,168]
	Radpour <i>et al.</i> (2011)	Gobel <i>et al.</i> (2011)	[160,169]
	Yamamoto <i>et al.</i> (2011)	Fujita <i>et al.</i> (2012)	[161,170]
	Lee <i>et al.</i> (2012)		[162]
	Sturgeon <i>et al.</i> (2012)		[163]
	Kloten <i>et al.</i> (2013)		[164]
	Chimonidou <i>et al.</i> (2013)		[165]
Colorectal	Grutzmann <i>et al.</i> (2008)	deVos <i>et al.</i> (2009)	[171,172]
	deVos <i>et al.</i> (2009)	Li <i>et al.</i> (2009)	[172,173]
	Li <i>et al.</i> (2009)	Philipp <i>et al.</i> (2012)	[173,177]
	Lange <i>et al.</i> (2012)		[174]
	Hibi <i>et al.</i> (2012)		[175]
	Church <i>et al.</i> (2013)		[176]
Cervical	Adebukadeer <i>et al.</i> (2012)		[178]
Gastric	[†] Zhai <i>et al.</i> (2012)	Balgkouranidou <i>et al.</i> (2013)	[108,179]
HCC	Chan <i>et al.</i> (2008)	Chan <i>et al.</i> (2008)	[60]
Liver	Sun <i>et al.</i> (2013)	Sun <i>et al.</i> (2013)	[180]
Lung	Lee <i>et al.</i> (2012)	Vinayanuwattikun <i>et al.</i> (2013)	[181,182]
Melanoma	Salvianti <i>et al.</i> (2012)	Hoshimoto <i>et al.</i> (2012)	[111,103]
	Lo Nigro <i>et al.</i> (2013)		[183]
Renal	de Martino <i>et al.</i> (2012)		[184]
Prostate	Ellinger <i>et al.</i> (2008)		[185]
	Sunami <i>et al.</i> (2009)		[110]
	Schwarzenbach <i>et al.</i> (2011)		[186]
	Dumache <i>et al.</i> (2012)		[187]
Ovary	Bondurant <i>et al.</i> (2011)		[188]
	Zhang <i>et al.</i> (2013)		[189]

[†]Article involving genome-wide or whole-genome analysis.

in DNAm profiles between primary tumor and cfDNAm was observed. Interestingly, their study also included the analysis of cfDNAm profiles of Barrett esophagus patients (n = 10), an esophageal cancer precursor and healthy controls (n = 10). They identified a cfDNAm signature that can distinguish pre-cancerous lesions from healthy controls and from cancerous lesions, however, to confirm the cancer-related cfDNAm profile, the number of patients should be increased.

The detection of cfDNAm represents an important non-invasive strategy for diagnosis and prognosis of cancer patients. However, several issues should be considered and addressed. Since the assay level of cfDNAm is the average of all the potential cfDNA sources, the methylation level of tumor-associated cfDNA can be masked by cfDNA from normal cells, resulting in a lack of sensitivity. This characteristic may require large tumor load to be detectable by cfDNAm-based approaches. Similarly, changes in DNAm patterns associated with non-cancerous processes, such as age, gender, race, medications, lifestyle, among other variables, need to be considered [98].

Detection of cfDNA using a multimarker approach

We have demonstrated that the use of multiple markers assays, based on diverse tumor alterations, can minimize false-positive or -negative results [109,110]. Multiple markers can deal with the tumor genetic and epigenetic heterogeneity and increase the sensitivity of cfDNA monitoring. As we have discussed in this review, different cfDNA characteristics provide valuable information about tumor burden; studies combining diverse diagnostic strategies have shown promising results. For example, Sunami *et al.* reported on the combination of genetic and epigenetic multiple markers assays to evaluate cfDNA from 81 prostate cancer patients [110]. cfDNA was assayed for allelic imbalance of six genome microsatellites and for DNAm of *RASSF1*, *RARB* and *GSTP1*. The combined assays detected prostate cancer in 15 of 24 patients (63%) with normal PSA concentrations regardless of AJCC stage. Combination of the multimarker cfDNA and PSA level assays increased the sensitivity from 70% (PSA alone) to 89%. Salvianti *et al.* also reported that an approach based on the simultaneous determination of three characteristics (total cfDNA,

Table 6. Ongoing and complete clinical trials focused on cfDNA analysis (patient cohort >50).

Organ	ClinicalTrials.gov identifier:	Phase	Responsible party	Source of cfDNA	Methods	Markers	Cohort No.	Start year
Brain	NCT00265174	N/S	Hadassah Medical Organization	Serum	Methylation, LOH	MGMT and LOH Profiling	60	2006
Brain	NCT00898391	N/S	Children's Oncology Group	Serum	Amplification	MYCN	329	2007
Breast	NCT00899548	N/S	Sidney Kimmel Comprehensive Cancer Center	Serum	Methylation	Genome-wide profiling	300	2006
Colorectal	NCT01212510	N/S	University Hospital, Rouen	Serum	Mt	N/A	500	2010
Colorectal	NCT01198743	N/S	Assistance Publique - Hôpitaux de Paris	Plasma	Mt and MSI	KRAS, NRAS, BRAF, APC and TP53	250	2005
Gastric	NCT01774266	N/S	Universidad Catolica de Chile	Serum	Methylation	Profiling	3000	2012
Lung	NCT01187290	Phase II	Shanghai Jiao Tong University School of Medicine	Serum	Quantification	N/A	80	2010
Lung	NCT00693836	N/S	Cancer Research Centre at Weston Park Hospital	Serum, plasma	Mt, LOH and allelotyping	Profiling	2000	2006
Lung	NCT00213798	N/S	University Hospital, Strasbourg, France	Plasma	Allelotyping	Profiling	200	Complete
Lung	NCT00290355	Phase II	GlaxoSmithKline	Plasma	LOH/MSI	Profiling	182	Complete
Lung	NCT01776684	N/S	Samsung Medical Center	Plasma	Mt	EGFR	200	2012
Lung	NCT01532089	Phase II	Academic and Community Cancer Research United	Plasma	Mt	EGFR	118	2012
Lung	NCT00997334	Phase II	Dana-Farber Cancer Institute	Plasma	Mt	EGFR	60	2009
Lung	NCT01785888	N/S	AstraZeneca	Plasma	Mt	EGFR	1300	2013
Lung	NCT01788163	Phase IV	AstraZeneca	Plasma	Mt	EGFR	3500	2013

cfDNA integrity index and cfDNAm) improved the diagnostic performance of the assay in melanoma [111]. The combination of multiple markers for cfDNA screening, each with limited sensitivity and/or specificity, can lead to a more robust screening test.

Clinical trials focused on tumor-associated cfDNA analysis

To truly estimate the clinical utility of cfDNA as a biomarker for cancer, the most conclusive results should be generated by defined clinical trials with well annotated specimens, involving large cohorts of patients and controls with multiples centers. Here, we explore ongoing and completed unpublished clinical trials focused on cfDNA.

We used the US National Health Institute registry to identify the most relevant characteristics of each clinical trial [201]. Currently, there are 21 ongoing and 6 complete registered clinical trials; 12 are focusing on lung, 7 gastrointestinal, 3 brain, 2 breast, 2 prostate and 1 pediatric cancer. In TABLE 6 we have listed 15 clinical trials, which include more than 50 patients, excluding 7 trials with a small patient cohort as well as 5 trials designed to collect and to store clinical specimens for later cfDNA studies. From a methodological point of view, eight are focused on mt, four on DNAm, three on LOH/MSI/allelotyping and one on cfDNA quantification analysis (FIGURE 3). Eight studies use cfDNA assessment as one of the primary endpoints and seven use it as one of the secondary

endpoints. We identified six major trials focused on the detection of *EGFR* mt on cfDNA from NSCLC; several of these studies include greater than 1000 patients (i.e., ClinicalTrials.gov identifiers: NCT01785888 and NCT01788163).

Remaining challenges

To ultimately evaluate the impact of cfDNA analysis, there is a need to perform more studies using a clinical trials setting, involving multiple centers, with a sufficient sample size of patients and good clinical annotation on disease progression. NGS technology brings a new alternative for unbiased cfDNA exploration. However, NGS studies have been performed in a very small cohort of patients and do not demonstrate adequate consistency yet. Thus, a remaining challenge is to expand these diagnostic alternatives to different tumor types and with larger sample size of patients and respective controls. Most importantly, it remains to be seen if cfDNA can stack up against existing approved blood cancer biomarkers. Do they improve patient analysis and provide more informative results of clinical status? Another challenge to cfDNA analysis in cancer patients is the low specificity. Issues also arise when cfDNA markers are not detected due to low sensitivity. cfDNA will, of course, be subjected to the rigorous analysis of biomarker standardization for approval.

This can only come about through significant resource investment by pharma/biotech-related companies. New approaches to cfDNA assessment will continue to be developed in the coming years; however, validation will remain a key issue. We often overestimate interpretations of new techniques on very small sample sizes of patients. The other major challenge is the sensitivity of cfDNA in detecting early stages of cancer or patients with small tumors. This has been a major issue for cfDNA as to whether the assays will be efficiently useful for all stages of cancer. Sampling volume and technical practice standards for extraction of cfDNA will be a continual challenge if the assays are to allow robust approaches for clinical testing. This has impaired the field over the years due, in part, to the limited amount of cfDNA in early stage cancer patients. Extraction procedures have not significantly improved in the past 5 years. Alternative approaches using direct non-extraction nucleic acid detection may be the way of the future, as we have previously demonstrated for various type of cancer [54,112]. With new

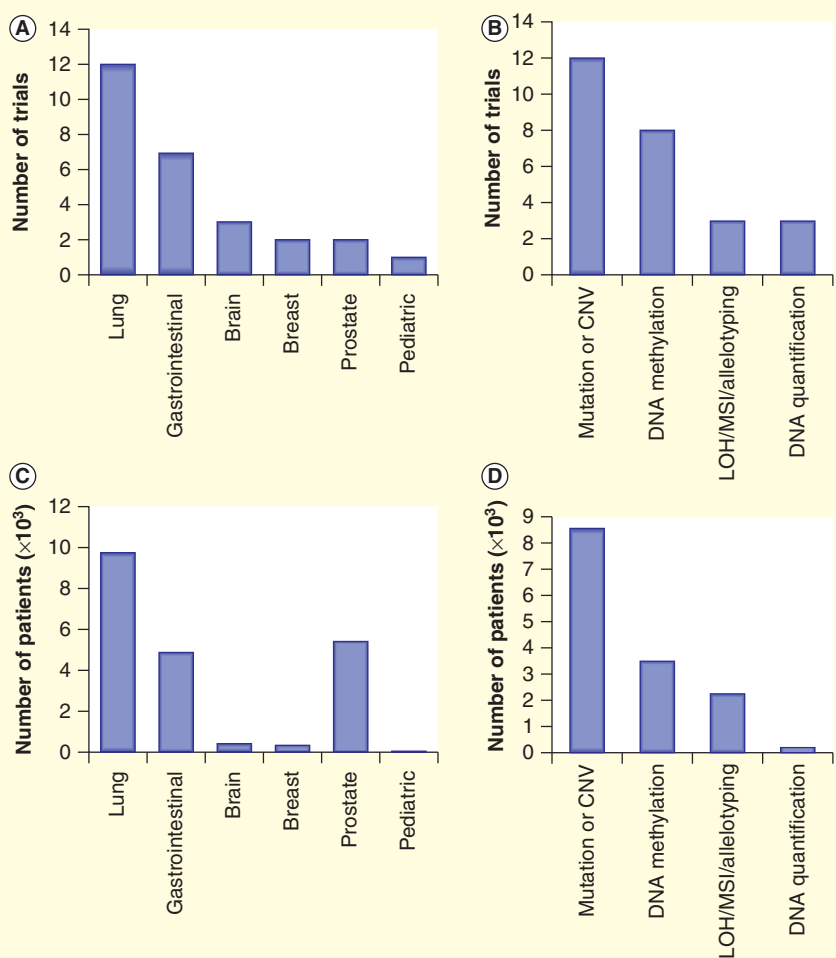


Figure 3. Trends in cancer types and technological approaches of ongoing and complete cfDNA clinical trials. (A) Number of trials per tumor type. **(B)** Number of trials using different molecular analysis. **(C)** Number of patients expected to be included in the trials per tumor type. **(D)** Number of patients expected to be included according different molecular analysis.

approaches in biosensors, microfluidics and new detection instruments in the coming years, it is suggested that exciting approaches will soon develop.

Expert commentary

The detection of cfDNA alterations provides a means with which patients at risk of relapse or tumor progression can be effectively identified as an alternative or adjunct to other non-blood-based approaches. This non-invasive (liquid biopsy) approach would allow the serial quantitative and qualitative cfDNA monitoring of primary and metastatic tumors to identifying changes pre- and post-treatment.

Given the challenges associated with inter- and intra-tumor heterogeneity and the clinical risk of multiple biopsies, the implementation of WGS of cfDNA will become the avenue to understand the tumor progression and therapeutic settings for both predominant and under-represented tumor subclones. Molecular-targeted therapies have shown promising results for

different tumor types. However, drug resistance is observed in the vast majority of the patients. As discussed in this review, the acquired resistance could be a consequence of pre-determined, under-represented subclones in the original tumor. Thus, the application of WGS of cfDNA can provide an alternative to identify, beforehand, resistant subclones and make more comprehensive and personalized treatment decisions. Whether WGS of cfDNA can be used for early detection has not been well studied in a large patient population. This is an expanding area; however, the cost of assay trade off with accurate detection level, still represents a problem to address.

Five-year view

With the advent of greater NGS resolution and the decrease in the cost per analysis, this technology has the potential to become the gold standard procedure for cfDNA screening in cancer patients. Among the most promising features of this technology is the increment of the detection specificity. The most important requirement for NGS clinical standardization with other approved blood-based biomarkers is to be tested in well-defined cohorts of patients in multicenter clinical trials.

Expeditious approval of cfDNA markers in CLIA and Food and Drug Administration settings is essential, otherwise the assays will have limited use and eventually be surpassed by other types of cancer biomarker assays. The field of research on blood cancer biomarkers has become critical recently because of the paucity of efficient biomarkers to monitor tumor progression during therapy. With the development of targeted genomic therapies, respective and specific cfDNA assays become more attractive as a surrogate for clinical monitoring.

Financial & competing interests disclosure

This work was supported by National Institute of Health; National Cancer Institute grant number PO1 CA029605 Project II and Core C to DSB Hoon; Ruth and Martin H. Weil Foundation (DSB Hoon); and the Leslie and Susan Gonda (Goldschmied) Foundation (Los Angeles, CA, USA). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- cfDNA has become increasingly used as a non-invasive diagnostic and prognostic liquid biopsy for cancer patients.
- cfDNA has been detected in plasma and serum of cancer patients as well as other pathological processes. It has been established that the level of cfDNA is generally higher in cancer patients than in healthy controls.
- In solid tumors, cfDNA is mainly released from non-programmed cell death such as necrosis. This process can be distinguished from apoptotic processes by assessing and comparing the length of the produced cfDNA fragments (DNA integrity index).
- Recently, the application of next generation sequencing to cfDNA analysis has provided a new alternative to profile tumor genomic alterations. This strategy allows the identification of under-represented tumor clones and inaccessible primary or metastatic tumors. The high cost and sensitivity of NGS remains a major challenge for its wide spread application.
- Detection of tumor-associated DNAm changes in cfDNA is a highly informative approach for both tumor biology and clinical application. It is imperative that this field expand to whole-genome approaches to elucidate more comprehensive DNAm landscapes.
- There is a need to evaluate cfDNA markers in large clinical trials. Currently, a small number of trials are focused on cfDNA, some of which include a large number of patients. However, to effectively evaluate the clinical utility of classical and NGS, an analysis of cfDNA is needed in the context of more and larger multicenter clinical trials.

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