Mol Diagn Ther (2015) 19:339–350
DOI 10.1007/s40291-015-0167-y

REVIEW ARTICLE

# Role of Circulating Cell-Free DNA in Cancers

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Published online: 23 September 2015


**Abstract** Liquid biopsy is a term used to describe non-invasive tests, which provide information about disease conditions through analysis of circulating cell-free DNA and circulating tumor cells from peripheral blood samples. In patients with cancer, the concentration of cell-free DNA increases, and structural, sequence, and epigenetic changes to DNA can be observed through the disease process and during therapy. Furthermore, cell-free DNA released by the tumor contains the same variants as those in the tumor cells. Therefore, cell-free DNA allows non-invasive assessment of cancer in real time. This review summarizes the origin of cell-free DNA, recent advancements in the detection of cell-free DNA, a possible role in metastasis, and its importance as a non-invasive diagnostic assay for cancer.

**Key Points**

Circulating cell-free DNA is present in different forms in the bloodstream.

Cell-free DNA has immense potential to be considered a diagnostic and prognostic marker.

Non-invasive procedures using cell-free DNA can be used to assess disease in real time.

## 1 Introduction

Mendel and Metais [1] first discovered cell-free nucleic acids in 1948. Later, they were observed in many disease conditions such as inflammatory and autoimmune diseases, lupus erythematosus, myocardial infarction, diabetes, and cancer [2, 3]. A higher concentration of cell-free DNA (cfDNA) was detected in cancer patients than in healthy individuals [3]. cfDNA levels reflect disease status [3–5], but the concentration alone is not sufficient to be used as a biomarker [6–8]. cfDNA released by the tumor contains the same mutations as those in tumor cells. Hence, cfDNA concentration and genetic alterations detected in cell-free DNA can, together, be used as cancer biomarkers. Biopsy of a tumor with intra- and inter-tumor heterogeneity does not provide a complete genetic picture of the tumor, as only a part of the tumor is tested. Furthermore, the diversity between primary tumors and metastatic tumors makes it difficult to highlight mutations, which can increase the metastatic ability of cancer cells. Circulating tumor DNA

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(ctDNA) includes DNA released not only by primary but also by metastatic tumors [9]. Therefore, it is possible to analyze ctDNA and obtain information about mutations present in primary and metastatic tumors [10]. Since procedures are non-invasive, testing can be repeated to follow the progress of disease or response to treatment [11, 12]. Mutations contributing to drug resistance can be detected, allowing therapeutic decisions to be changed accordingly [13–16]. Hence, ctDNA has immense potential as a cancer biomarker. Inclusion of non-invasive ctDNA assays would be helpful in cancer diagnostics, assessing disease status, and monitoring treatment response. This review summarizes the origin of cfDNA, recent advancements in the detection of cfDNA, a possible role in metastasis, and its importance as a non-invasive diagnostic assay for cancer.

## 2 Types of Cell-Free DNA (cfDNA)

cfDNA are present in various forms such as unbound DNA fragments, nucleosomes, vesicle-bound DNA, and virtosomes (Fig. 1) [17].

### 2.1 DNA Fragments

Free DNA fragments are not bound to any other molecule or surface. Upon release, they are digested by DNases in blood. However, in cancer, the DNase concentrations are low and DNase inhibitors have also been detected, which could also contribute to increasing levels of cfDNA [18]. Circulating DNA has been reported to have a half-life of less than 2 h [19, 20].

### 2.2 Nucleosomes

Nucleosomes are complexes consisting of DNA wrapped around an octamer of histone proteins and are stabilized by histone H1. Nucleosomes are linked to each other by 100 bp of linker DNA. During apoptosis, chromatin is cleaved into oligo- and mono-nucleosomes, packed into vesicles, and released from the cell before being engulfed by neighboring cells. In cancer, during chemotherapy or radiotherapy, the rates of cell death are high, and the phagocytic mechanism becomes saturated, leading to increased levels of nucleosomes in circulation [4]. Increased nucleosome concentrations correlated with cfDNA concentrations; this has been linked to metastasis and disease progression in breast cancer [21]. Circulating nucleosome levels have been shown to predict therapeutic efficacy in lung cancer, acute myeloid leukemia, colorectal cancer (CRC), and pancreatic cancer [22–25].

### 2.3 Vesicle-Bound DNA

Vesicle-bound DNA include DNA contained in apoptotic bodies, exosomes, and microparticles [17]. Apoptotic

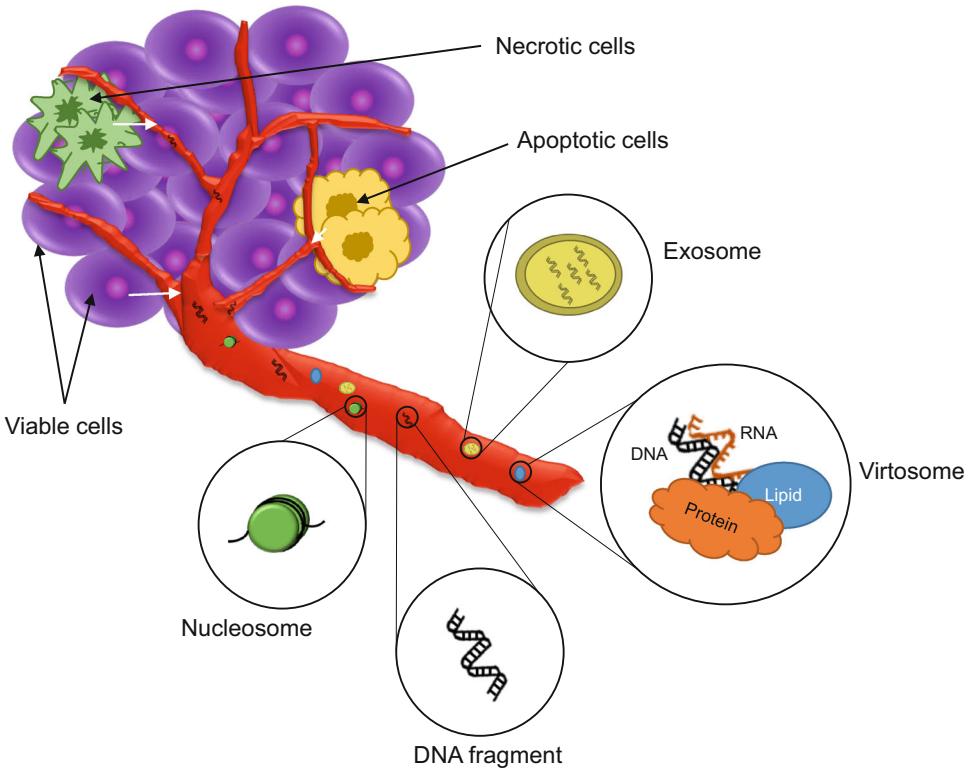


**Fig. 1** Circulating cell-free DNA. DNA can be released into the bloodstream either through cell death, i.e. apoptosis (*yellow*) or necrosis (*green*) or it can be released by viable cells (*purple*). Cell-free DNA can be present in the form of unbound DNA, nucleosomes, vesicle-bound DNA, or virtosomes

bodies are formed by cell blebbing during apoptosis. Nucleic acids are then packaged in these bodies and released from the cell to be ingested by phagocytes. Holmgren et al. [26] showed that apoptotic bodies can be taken up by phagocytic cells, causing horizontal transfer of DNA. Exosomes are small vesicles produced by many cell types. They may contain nucleic acids as well as protein. Exosomes released by tumor cells contain mutated nucleic acid fragments that may contribute to the growth and spread of primary and metastatic cancers [27].

### 2.4 Virtosomes

Virtosomes are complexes of newly synthesized DNA, RNA, proteins, and lipids and are only released by living cells [17, 28]. The nucleic acids cannot be readily digested with nucleases, unless the complex is first treated with proteinase or lipase [28]. The protein portion consists of DNA: dependent DNA and RNA polymerases [17, 28]. The complex is released in a controlled energy-dependent manner [17, 28]. The DNA is synthesized in nucleus and moves into cytosol where it is joined by newly synthesized protein, lipid, and RNA before being released from the cell [28]. Virtosomes can easily enter and change the biology of recipient cells. Hence, virtosomes released by cancer cells may enter and transform normal cells [28].

## 3 Origin

Although the origin of ctDNA is not clear, several studies have proposed that ctDNA could be released by apoptosis or necrosis or shed by viable tumor cells (Fig. 1). Jahr et al. [29] investigated the origin of plasma cfDNA using size distribution analysis. Although the size of DNA was frequently in the range of multiples of 180 bp, similar to DNA from apoptotic cells, DNA fragments of high molecular weight, similar to necrotic cell DNA, were also detected in some samples. Chromatin fragments were observed to be released by cells undergoing induced cell death in animal models and were eventually detected in the bloodstream of the models. Therefore, ctDNA may be released by dying tumor cells [29]. A mechanism of cfDNA release was proposed by Diehl et al. [30], whereby invasive tumors that outgrow their blood supply contain larger regions of necrosis than benign tumors. The necrotic cells are then engulfed by macrophages that release digested DNA into the surrounding medium. Active release by lymphocytes has also been viewed as a possible origin of cfDNA [31]. Evaluations of associations between cfDNA and alterations in the balance of angiogenesis activators and inhibitors have suggested that cfDNA may reach plasma at least in part due to matrix metallopeptidase 2 (MMP2) overexpression [32].

## 4 Genometastasis

In 1999, García-Olmo et al. [31] proposed that ctDNA containing dominant oncogenes could transform susceptible cells in distant organs, thereby contributing to metastasis of tumors. They termed this process ‘genometastasis’ (Fig. 2). Rat colon cancer cell line DHD/k12-PROb cultured in plasma from tumor-bearing rats showed that the cfDNA may have properties for integrating into host cell genome [33]. Furthermore, horizontal transfer of DNA may occur via uptake of apoptotic bodies or virtosomes by phagocytic cells [26, 28]. In three different studies, when immortalized and normal cells were cultured in patients’ plasma, immortalized cells were transformed into cancer cells while normal cells were not transformed [34–36]. No transformation was seen when cell lines were exposed to normal plasma [36]. Therefore, ctDNA may play a role in cancer metastasis through oncogenic transformation of initiated cells.

## 5 Methods for Detection of cfDNA

### 5.1 Quantitative Polymerase Chain Reaction (PCR)

Quantitative real-time polymerase chain reaction (PCR) enables the evaluation of nucleic acid concentration by measuring the emitted signal from a fluorescent-labelled probe during amplification of the target gene [37]. Amplification of various genes such as *human telomerase reverse transcriptase* (*hTERT*) and *beta globin* have been used to measure cfDNA concentration [5, 38]. Furthermore, some studies used real-time PCR to measure DNA integrity [39, 40].

Methylation-specific PCR is an assay for determining the status of CpG islands in which bisulfite modification is performed followed by PCR amplification of the gene of interest using primers specific for either a methylated or an unmethylated DNA sequence [41]. A combination of real-time PCR and methylation-specific PCR was used to determine the methylation status of various genes and to quantitate methylated cfDNA sequences [42–44].

In digital PCR, DNA is serially diluted to isolate single molecules; each molecule is then individually analyzed for mutations [45]. Digital PCR was used to screen for alterations in *PIK3CA* mutations in breast cancer [12]. Sensitivity of this technique was 93.3 % and specificity was 100 % [12]. Screening of *HER2* copy number was carried out in patients with breast cancer using digital PCR [46]. The positive predictive value was 70 % for this assay, and the negative predictive value was 92 % [46]. *KRAS* and *BRAF* variants were detected using duplex digital PCR in patients with CRC [47]. Exon 19 deletion and L858R

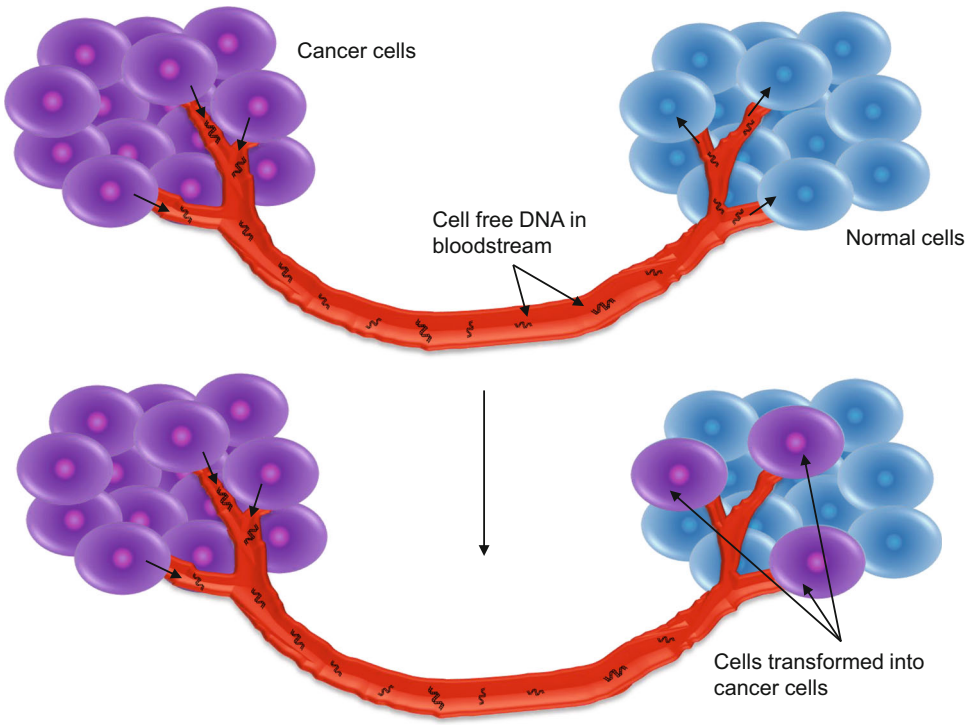


**Fig. 2** Genometastasis. Circulating DNA has been suspected has having a role in metastasis. Studies in this direction have shown that circulating tumor DNA has the ability to transform susceptible cells in distant organs

mutation were detected in *EGFR* gene in lung cancer patients with 92 % sensitivity and 100 % specificity [14].

### 5.2 Next-Generation Sequencing

Next-generation sequencing (NGS) has been used to analyze ctDNA in many cancer types to identify mutations. Leary et al. [48] devised a technique called 'personalized analysis of rearranged ends' (PARE) to detect chromosomal rearrangements in solid tumors that were then used to develop biomarkers for the monitoring of tumor through plasma DNA. However, limitations of this technique include dependence of sensitivity on amount of sequence data, cost, possibility of detecting false positives, and the unavailability of information on source of ctDNA from these analyses [49].

De Mattos-Arruda et al. [10] used massive parallel sequencing to analyze DNA extracted from primary and metastatic tumors and plasma samples from a 66-year-old patient with breast cancer. A total of 15 mutations were detected in both the primary and metastatic tumors, while two mutations were detected in metastatic tumors but not in the primary tumor. All of these variants were detected in ctDNA, thereby capturing the heterogeneity of tumors. Furthermore, on monitoring the mutant allele fractions of ctDNA during treatment, it was found that ctDNA analysis gave an earlier indication of disease progression than radiologic and biochemical assessments [10]. In another study, massive parallel sequencing of circulating cfDNA was used to study metastatic cancers in patients receiving treatment. It was observed that mutant alleles increased with development of therapy resistance [16].

Newman et al. [50] developed a new technique called cancer personalized profiling by deep sequencing (CAPP-SEQ) for quantifying ctDNA. They designed a probe panel to detect recurrently mutated genes using whole exome sequencing data for lung cancer patients from The Cancer Genome Atlas (TCGA). The panel targets included 521 exons and 13 introns from 139 recurrently mutated genes. They identified ctDNA in 100 % of stage II–IV non-small cell lung cancer (NSCLC) patients, with 96 % specificity for mutant allele fractions as low as 0.02 % [50]. Rothé et al. [51] evaluated plasma as an alternative for tissue biopsies with NGS of plasma DNA and tumour DNA. Mutations were detected in *p53*, *PIK3CA*, *PTEN*, *AKT1*, *IDH2*, and *SMAD4* genes [51]. In two samples, mutations were detected in tumor but not in plasma. This may have been due to the presence of a mutation allele frequency below the detection limit of the method used. Two samples showed mutations in plasma but not in tumor. These mutations may have been present in tumour clones that were not captured by biopsy [51].

Whole genome sequencing of cfDNA has been reported to detect novel mutations not detected by targeted sequencing [52]. However, whole genome sequencing is expensive and requires expertise in analysing the enormous amount of data it generates. Targeted sequencing is limited to mutations in genes. Sequencing of genes/exonic regions

**Table 1** Comparison of different methods available for cell-free DNA analysis

| Methods | Advantages | Disadvantages |
|---|---|---|
| Quantitative PCR | Wide choice in detection chemistry and reaction volume equates to flexible running costs | Fewer genes can be studied at a given time |
| | Larger number of samples can be analyzed | |
| | Large dynamic range | |
| | Relative measurement | |
| Digital PCR | High precision for better reproducibility for low-input target concentrations | Initial cost of equipment |
| | Higher precision for higher copy number variation analysis | Pre-amplification of target DNA on samples may result in biased amplification of input DNA |
| | Greater sensitivity for rare mutation detection | |
| | Absolute measurement eliminates need for standard curve | |
| Next-generation sequencing | | |
| Targeted sequencing | Permits large number of genes to be studied in large number of samples (multiplexing possible) | Sensitivity depends on depth of sequence coverage |
| | Specific genomic region/genes can be analyzed | Possibility of detecting false positives |
| | | Higher cost |
| Exome sequencing | Clinically relevant information from cell-free DNA point of view | Higher input material is required; higher cost implication |

*PCR* polymerase chain reaction

of a gene that are frequently mutated in individual cancers may help circumvent the problem.

### 5.3 BEAMing

Diehl et al. [30] developed BEAMing (beads, emulsion, amplification, and nagnetics) to detect somatic mutations in DNA. In this technique, the DNA segment of interest is first amplified using primers containing known tag sequences. Emulsion PCR amplifies the DNA sequences again using primers targeting the tags. The DNA sequences are then covalently bound to magnetic beads. Labelled nucleotides are added to the sequences by single base extension, and flow cytometry is used to sort beads containing mutant allele from those containing wild-type allele [30]. The technique is highly sensitive, with the ability to detect and measure DNA even when there is one mutated DNA present in 10,000 DNA molecules [53]. BEAMing was used to detect *APC* variants in CRC [30], *EGFR* variants in lung cancer [53], and *PIK3CA* variants in breast cancer [54].

### 5.4 Peptide Nucleic Acid Clamp-Mediated PCR

Peptide nucleic acid (PNA) is a synthetic nucleic acid analog with a peptide backbone consisting of *N*-(2-aminoethyl)-glycine units, instead of phosphodiester backbone [55]. In PNA-mediated PCR reactions, PNA complementary to wild-type sequence acts as a clamp. Mismatch of one base pair can distinguish between a wild-type sequence and a mutated sequence. Hence, PNA binds specifically to wild-type sequences, preventing amplification, while mutated sequences are amplified using primers [55]. *EGFR* variants were identified using PNA clamp-mediated PCR in NSCLC patients. The mutation detection rate correlated with sex, smoking history, and stage. However, this method was limited in terms of its sensitivity and the multiple steps, lasting several days [56]. Däbritz et al. [57] combined PCR clamping and melting curve analysis to detect *KRAS* codon 12 variants in pancreatic cancer patients. The sensitivity of the assay was 1–5 $\times$ $10^5$; 28 % of plasma samples were found to have alterations in the *KRAS* gene. The most common alteration was glycine to valine, seen in 83 % samples [57].

Even though techniques such as quantitative PCR and BEAMing are sensitive, they require prior knowledge of the variants present in the patient (Table 1). NGS methods do not require this. Different panels can be used to screen for mutations in multiple genes. This would enable the detection of various cancer-associated genetic alterations in patient blood. Therefore, sequencing circulating tumor DNA using NGS techniques would be useful in the non-invasive detection of genetic alterations.

## 6 cfDNA: A Non-Invasive Biomarker

cfDNA has many clinical applications as a diagnostic, predictive, and prognostic biomarker for cancer (Fig. 3). The presence and concentration of somatic sequence

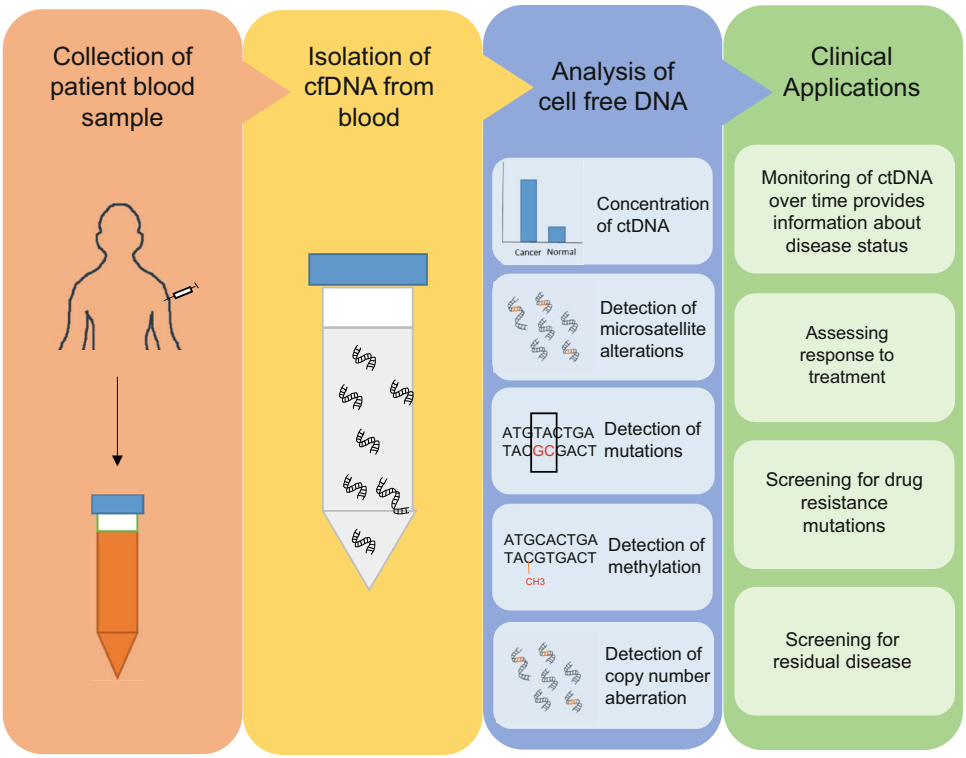


**Fig. 3** Clinical applications of circulating tumour DNA. Circulating tumour DNA is easily accessible as it is obtained from the peripheral blood of patients. It is being explored as a potential biomarker for cancer through the measurement of circulating tumor DNA concentration and detection of genetic alterations such as mutations, methylation, and microsatellite alterations. Monitoring these parameters in periodically collected blood samples would give information about disease progression and treatment response. It also allows screening for drug-resistance mutations, thereby aiding in treatment decision making. Minimal residual disease can be detected with the evaluation of cell-free DNA. *cfDNA* cell-freeDNA, *ctDNA* circulating tumor DNA

variants, changes in methylation status, copy number variants, and microsatellite instability provide data on disease status. As cfDNA changes with disease status during therapy, periodic assessment can be carried out to evaluate disease progression and treatment response. Furthermore, variants in tumor DNA can contribute to drug resistance. Analysis of ctDNA for such variants may aid in deciding the appropriate treatment for the patients.

### 6.1 Concentration

cfDNA levels have been measured via many different techniques such as radioimmunoassay, DNA dipstick, and real-time PCR in various types of cancers [3, 5, 58, 59]. The levels reported vary across different studies, but cfDNA concentration is generally higher in cancer patients than in healthy individuals [3–5, 21, 58–61]. A comparison of different cancer types showed that patients with lung cancer had the highest levels of nucleosomes, and prostate cancer patients had the lowest levels [4].

Comparisons of median cfDNA concentrations in plasma and serum showed cfDNA levels were higher in serum than in plasma in both patients and healthy controls [62]. However, this could be due to the release of DNA by cell lysis during clotting. Plasma DNA levels correlated with tumor size [63], degree of tumor invasion [3], disease stage, survival, and disease progression under therapy [3, 5, 13, 62]. Increased cfDNA levels were associated with aggressive disease and worse prognosis in lymphoma [38, 61]. Nucleosome concentrations correlated with circulating DNA concentrations and caspase activities in breast cancer [21]. Increased levels of serum nucleosomes, cfDNA, and protease activities were related to metastasis and hence may be linked to progression of breast cancer [21]. Patients with metastatic cancer had higher ctDNA levels than those with non-metastatic disease [3].

cfDNA levels were observed to decrease after therapy or surgery [3, 11, 58]. During chemotherapy nucleosomes levels increased initially and later decreased; while following tumour resection it decreased soon after surgery [4, 60, 64]. This could be due to the clearance of nucleosomes from circulation [64]. The post-therapeutic concentration of circulating nucleosomes correlated with response to treatment [4, 64]. Decreased ctDNA levels correlated with improved clinical condition, while increased or unchanged levels indicated a lack of response to treatment [3]. Furthermore, levels were lower in disease-free individuals than in patients experiencing relapse [58]. While no cases have been observed showing the presence of circulating tumor cells (CTCs) with an absence of ctDNA, a ctDNA presence has been found in patients with an absence of CTCs [13]. In some cases, ctDNA concentration was more than fiftyfold that of CTCs. [13]. One study has reported an instance in which a patient had large numbers of CTCs but low levels of cfDNA [65].

Many groups have reported cfDNA concentration to be very high in malignant cancer, moderately high in benign

disease, and low in healthy individuals [66–68]. However, few studies have reported cases in which DNA concentration alone is not sufficient to discriminate between benign and malignant cases [6, 7, 21]. For instance, in one study, plasma DNA concentration measured using real-time PCR were not significantly different between healthy individuals and those with gastroesophageal reflux disease or esophageal and lung cancer [7]. It has also been reported that some patients with highly metastatic cancer may not have detectable amounts of plasma DNA [9]. The techniques used and concentrations measured also vary across studies. Hence, it is difficult to establish a cut-off value to distinguish between healthy, benign, and malignant conditions. However, as cfDNA levels reflect the progression of disease and effect of therapy [5], they can be used to monitor disease status combined with other parameters such as mutations in ctDNA and DNA integrity.

### 6.2 DNA Integrity

The DNA integrity index (DII) is determined by amplifying DNA fragments of different sizes by PCR and calculating the ratio between concentrations of long PCR products and concentrations of short PCR products. Many studies have tried to establish an appropriate DII ratio to distinguish between cancer patients, those with benign disease, and healthy individuals.

Different studies have suggested different integrity indices as discriminating values. A 300/60 ratio was suggested as an optimal value in one study [40]. Pinzani et al. [39] compared DII ratios of 180/67, 306/67, and 476/67 in cutaneous melanoma patients. All ratios showed higher values in cancer patients. Although a combination of the three values gave high sensitivity, the 180/67 value was found to be the most suitable by receiver operating characteristic (ROC) curve analysis [39].

The detected ctDNA concentration inversely correlated with amplicon size [30]. The size distribution of neoplastic cfDNA differs from that of non-neoplastic cfDNA [40]. Some studies have reported tumor cfDNA to be more fragmented than normal cfDNA [30, 69]; however, DNA integrity has also been reported to be higher in patients than in healthy controls [39, 40]. Jiang et al. [70] studied the size profile of cfDNA using massive parallel sequencing in patients with hepatocellular carcinoma (HCC). They observed that short as well as long fragments of cfDNA were present in patient plasma. The short fragments were also observed to contain more copy number aberrations [70].

The size distribution of cfDNA has been observed to be biphasic in cancer patients [9]. The biphasic size distribution also correlated with CTCs and levels of mutated DNA fragments [9]. DNA integrity was observed to be more representative of tumor progression than absolute serum DNA values [71]. DNA integrity was higher in patients positive for lymphovascular invasion, micrometastasis, or lymph node metastasis than in negative patients [71]. In patients with post-operative recurrence of disease, serum DNA integrity was similar to that of patients with stage III or IV breast cancer. Evaluation of serum DNA integrity achieved 69 % sensitivity with 80 % specificity for detecting stage II–IV breast cancer and sensitivity of 74 % with 80 % specificity for lymph node metastasis [71].

### 6.3 Genetic Alteration

In cancers, accumulation of genetic alterations occurs in genes that are involved in cellular processes such as growth and apoptosis. Genetic alterations detectable in tumors can also be detected in cfDNA [72]. Detection of cancer-related mutations in cfDNA indicated that the cfDNA was tumor derived [2]. Since ctDNA may be released by primary as well as by metastatic tumors, analysis of ctDNA may present a whole paradigm of various mutations present in primary as well as in metastatic tumors [10]. Furthermore, monitoring the mutant allele fractions of ctDNA during treatment can give an earlier indication of disease progression than radiologic and biochemical examinations [10]. Tumor cells in cancer patients acquire variants that make them resistant to the effects of chemotherapeutic drugs. Therefore, patients being considered for treatment with such drugs should be tested for drug-resistance mutations, as these would otherwise compromise the efficacy of the treatment.

#### *6.3.1 Mutations*

*EGFR* variants have been detected in ctDNA in various cancers. Detection of *EGFR* mutation in tumor tissue and plasma was 94.3 % concordant, with 99.8 % assay specificity and 65.7 % sensitivity [73]. The concentration of mutated sequence in plasma correlated with response to treatment. Patients showing complete or partial remission had decreased concentrations, while increased concentrations indicated no response [14]. Furthermore, *EGFR* mutation re-emerged in a patient whose treatment was stopped after an initial response [14]. Detection of mutated DNA fragments in plasma can predict the response to treatment. These data indicate that monitoring ctDNA during the treatment course can present real-time information on patient response to treatment [74]. The presence of *EGFR* mutations has correlated with response to gefitinib and progression-free survival time. *EGFR* variants were detected more frequently in patients who responded to gefitinib and in those with longer survival [75]. Emergent variants were identified in 96 % of patients who

initially responded to *EGFR* blockade treatment and later progressed [13].

The *KRAS* gene is also involved in the EGFR pathway; it has been studied in CRC and pancreatic cancer. p.Gly12Ala, p.Gly12Cys, p.Gly12Ser, p.Gly12Asp, p.Gly12Val, p.Gly12Arg, and p.Gly13Asp are some of the variants detected in codons 12 and 13 of the *KRAS* gene [47, 76]. Mohan et al. [52] found that *KRAS* gene amplifications in ctDNA correlated with the clinical course of disease. Mutations and amplifications in the *KRAS* gene are mutually exclusive. However, they also observed that *KRAS* amplification may occur during *EGFR*-targeted treatment of CRCs [52]. The sensitivity of a liquid biopsy for the presence of variants in codons 12 and 13 of the *KRAS* gene was 87.2 %, and specificity was 99.2 % [13]. The proportion of the mutated alleles seems to be highly variable, ranging from <1 to 64 %. This would have implications with regard to the technique to be used for the most sensitive detection. *PIK3CA* variants have also been detected in plasma post-operatively in patients who showed no clinical symptoms [12]. Hence, cfDNA can be used to detect minimal residual disease.

### 6.3.2 Microsatellite Instability

Microsatellites are DNA sequences in which a short motif is repeated many times. They are subject to expansion and contraction due to defects in mismatch repair machinery and can be used to detect loss of heterozygosity (LOH) in cancer [77]. They were first reported in head and neck cancer and lung cancer in 1996 [78, 79]. Microsatellite alterations were associated with disease status and patient survival in lung cancer [5] and melanoma [80, 81]. In oral squamous cell carcinoma, the presence of microsatellite alterations on chromosomes 2, 3, and 21 in serum DNA correlated with poor prognosis [82, 83].

### 6.3.3 Loss of Heterozygosity

The detection rate of LOH in chromosomes 1p, 19q, and 10q and methylation status of *MGMT* and *PTEN* gene promoters in serum DNA in high-grade astrocytomas or oligodendrogliomas were 51 and 55 %, respectively, with 100 % specificity [2]. LOH on chromosomes 6q, 8p, 9p, 10p, and 18q and DNA methylation correlated with Gleeson score and prostate-specific antigen (PSA) levels in prostate cancer. A combination of DNA assays and a PSA assay gave a sensitivity of 89 % [84]. Microsatellite alterations were detected in DP1 (D5S346) on 5, 486 (D7S486) on 7q, 522 (D7S522) on 7q, D11 (D11S904) on 11p, *p53V* (intron1 in p53) on 17p, and *BRCA1* (D17S855) on 17q in ovarian cancer patients [85]. High clonality was found between most serum and peritoneal fluid and their corresponding tumor samples. A higher frequency of novel alleles were found in early-stage patients than in later-stage patients [85].

### 6.3.4 Methylation

Methylation is an event in which a methyl group is added to the carbon at the fifth position of a cytosine ring, resulting in the formation of a 5-methylcytosine [86]. DNA methylation helps in the epigenetic regulation of gene expression. In cancer, genes that could inhibit tumorigenesis are often hypermethylated to stop their expression [86]. Several groups have studied the methylation status of such genes in cfDNA [87–90].

*P16* was found to be aberrantly methylated in breast cancers [91], HCC [87, 89], NSCLC [92], esophageal squamous cell cancer [93], and CRC [94]. In lymphoma, *p16* methylation co-occurred with fragmented DNA in plasma, and both parameters correlated with disease stage [95]. After surgical resection of HCC, the median plasma *p16* methylation reduced 12-fold [87]. *P16* and *p15* was concurrently methylated in HCC [96] and head and neck squamous cell carcinoma (HNSCC) [97]. A panel of genes including *p16, DAPK*, *GSTP1*, and *MGMT* was methylated in lung cancer [98] and head and neck cancer [99, 100]. *APC* was aberrantly methylated in lung cancer [101].

In breast cancer patients undergoing neo-adjuvant therapy, serum *RASSF1A* methylation became undetectable during therapy in patients achieving response but persisted in patients who had partial or minimal response [102]. *RASSF1A* methylation significantly correlated with overall survival and chemotherapy response in melanoma patients [103]. Methylated *RASSF1A* and/or *APC* genes in serum DNA were independently associated with poor outcomes in breast cancer [104]. Methylation of *APC, RASSF1A, GSTP1*, and *ESR1* correlates with the presence of CTC in breast cancer [105, 106]. *RASSF1A* methylation was also detected more frequently than *APC* and *ESR1* [105]. The presence of methylated *APC*, *RASSF1A* genes, and CTCs correlated with American Joint Committee on Cancer (AJCC) staging. Methylated *GSTP1* was mainly detected in patients with large primary tumors and was highly correlated with positive human epidermal growth factor receptor (Her)-2/neu status [106].

The presence of high amounts of methylated DNA sequences and CTCs correlated with a more aggressive tumor biology and advanced disease [105, 106]. Hypermethylation of *GSTP1* and *RARB2* was detected only in stage IV patients with prostate cancer, indicating that these two may have roles in invasion and metastasis [84]. Methylation analysis of *RARB2* and *RASSF1A* provided 95 % diagnostic coverage in breast cancer patients and 60 % in patients with benign lesions [107]. *RASSF1A* gene

promoter methylation was more frequently detected in samples of glial tumors than in metastatic central nervous system (CNS) cancers [88]. Hence, methylated genes can be used to distinguish between malignant and benign cases [88, 107]. Methylation of *APC*, *RARB2*, and *RASSF1A* was detected more frequently in patients with advanced disease [44].

Alpha-feto protein (AFP) levels have been used as a biomarker for HCC [108]. However, since it has low diagnostic sensitivity, Chan et al. [43] evaluated the use of serum methylated *RASSF1A* sequences for HCC diagnosis and prognosis. Serum *RASSF1A* correlated with the presence of cirrhosis in hepatitis B virus (HBV) carriers but not in HCC patients. The diagnostic sensitivity and specificity for the combined analysis of *RASSF1A* methylation and AFP levels were 77 and 89 %, respectively, compared with 65 and 87 %, respectively, for AFP measurement alone. Hence, combined *RASSF1A* and AFP testing may allow early diagnosis of HCC [43].

Hypermethylated *HLTF* and *HPP1* genes were detected frequently in patients with CRC with increased lactate dehydrogenase (LDH) levels [109]. Methylation of *HLTF*, *HPP1*, and *HMLH1* were associated with tumor size, advanced disease stages, and shorter survival in the overall population [109, 110]. Methylation of *HLTF* and *HPP1* was strongly correlated with cell death [109]. Methylation analysis of *KIF1A*, *DCC*, *NISCH*, and *RARB* genes was able to differentiate patients with cancerous tumors from those with non-cancerous tumors with 71 % specificity and 73 % sensitivity. However, each gene was less sensitive as independent assays [111]. *XAF1* gene was hypermethylated in 69.8 % of gastric cancer serum samples. Methylation of *XAF1* gene was a predictor of poor survival and associated with tumor recurrence [112]. Methylation of *ATP4B* gene was detected in the plasma of patients with gastric cancer [113].

## 7 Conclusion

Analysis of cfDNA from patient blood samples to obtain information about their disease status represents a convenient method of disease monitoring. Assessment of prognostic significance using cfDNA assay is non-invasive and enables simpler periodic assessment. As it provides information on each patient's disease status in real-time, treatment decisions can be made according to the patient's condition. An appropriate panel of genes can be used to screen for gene alterations for cancer diagnosis and prognosis, and for detection of drug-resistance mutations. Development of such cfDNA-based assays for clinical application would transform the current concepts of cancer diagnosis and prognosis and the assessment of therapeutic response. Future research work needs to focus on better and cost-effective isolation techniques for cfDNA, more robust detection of the spectrum of mutations in the cfDNA, and should compare primary tumor heterogeneity with mutations seen in cfDNA to assess the role of cfDNA as a better sample for molecular analysis than core biopsy or fine needle aspiration cytology.

**Compliance with Ethical Standards**

The authors, Raghu Aarthy, Samson Mani, Sridevi Velusami, Shirley Sundarsingh, and Thangarajan Rajkumar, have no conflicts of interest to declare. This review was supported by the Department of Science and Technology, Govt. of India [No. SR/S9/Z-08/2010 dated 25.06.2010].

## References

1. Mandel P, Metais P. Les acides nucleiques du plasma sanguin chez l'homme. C R Acad Sci Paris. 1948;142:241–3.
2. Lavon I, Refael M, Zelikovitch B, Shalom E, Siegal T. Serum DNA can define tumor-specific genetic and epigenetic markers in gliomas of various grades. Neuro Oncol. 2010;12:173–80.
3. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977;37:646–50.
4. Holdenrieder S, Stieber P, Bodenmüller H, Busch M, Fertig G, Fürst H, et al. Nucleosomes in serum of patients with benign and malignant diseases. Int J Cancer (Pred Oncol). 2001;95:114–20.
5. Sozzi G, Conte D, Mariani L, Lo Vullo S, Roz L, Lombardo C, et al. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. Cancer Res. 2001;61:4675–8.
6. Chang H-W. Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. Cancer Spectrum Knowl Environ. 2002;94:1697–703.
7. Herrera LJL, Raja S, Gooding WE, El-Hefnawy T, Kelly L, Luketich JD, et al. Quantitative analysis of circulating plasma DNA as a tumor marker in thoracic malignancies. Clin Chem. 2005;51:113–8.
8. Roth C, Kasimir-Bauer S, Pantel K, Schwarzenbach H. Screening for circulating nucleic acids and caspase activity in the peripheral blood as potential diagnostic tools in lung cancer. Mol Oncol. 2011;5:281–91.
9. Heitzer E, Auer M, Hoffmann EM, Pichler M, Gasch C, Ulz P, et al. Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer. Int J Cancer. 2013;133:346–56.
10. De Mattos-Arruda L, Weigelt B, Cortes J, Won HH, Ng CKY, Nuciforo P, et al. Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. Ann Oncol. 2014;25:1729–35.
11. Schwarz AK, Stanulla M, Cario G, Flohr T, Sutton R, Möricke A, et al. Quantification of free total plasma DNA and minimal residual disease detection in the plasma of children with acute lymphoblastic leukemia. Ann Hematol. 2009;88:897–905.
12. Beaver JA, Jelovac D, Balukrishna S, Cochran RL, Croessmann S, Zabransky DJ, et al. Detection of cancer DNA in plasma of early-stage breast cancer. Clin Cancer Res 2014;20:2643–50.
13. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6:224ra24.

14. Yung TKF, Chan KCA, Mok TSK, Tong J, To K-FF, Lo YMD. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. Clin Cancer Res. 2009;15:2076–84.
15. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res. 2014;20:1698–705.
16. Murtaza M, Dawson S-J, Tsui DWY, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013;497:108–12.
17. Peters DL, Pretorius PJ. Origin, translocation and destination of extracellular occurring DNA—a new paradigm in genetic behaviour. Clin Chim Acta. 2011;412:806–11.
18. Tamkovich SN, Cherepanova AV, Kolesnikova EV, Rykova EY, Pyshnyi DV, Vlassov VV, et al. Circulating DNA and DNase activity in human blood. Ann N Y Acad Sci. 2006;1075:191–6.
19. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14:985–90.
20. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet. 1999;64:218–24.
21. Roth C, Pantel K, Müller V, Rack B, Kasimir-Bauer S, Janni W, et al. Apoptosis-related deregulation of proteolytic activities and high serum levels of circulating nucleosomes and DNA in blood correlate with breast cancer progression. BMC Cancer. 2011;11:4.
22. Mueller S, Holdenrieder S, Stieber P, Haferlach T, Schalhorn A, Braess J, et al. Early prediction of therapy response in patients with acute myeloid leukemia by nucleosomal DNA fragments. BMC Cancer. 2006;6:143.
23. Kremer A, Wilkowski R, Holdenrieder S, Nagel D, Stieber P, Seidel D. Nucleosomes in pancreatic cancer patients during radiochemotherapy. Tumour Biol. 2005;26:44–9.
24. Kremer A, Holdenrieder S, Stieber P, Wilkowski R, Nagel D, Seidel D. Nucleosomes in colorectal cancer patients during radiochemotherapy. Tumour Biol. 2006;27:235–42.
25. Holdenrieder S, Stieber P, von Pawel J, Raith H, Nagel D, Feldmann K, et al. Circulating nucleosomes predict the response to chemotherapy in patients with advanced non-small cell lung cancer. Clin Cancer Res. 2004;10:5981–7.
26. Holmgren L, Szeles A, Rajnavölgyi E, Folkman J, Klein G, Ernberg I, et al. Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood. 1999;93:3956–63.
27. Tickner JA, Urquhart AJ, Stephenson S-A, Richard DJ, O'Byrne KJ. Functions and therapeutic roles of exosomes in cancer. Front Oncol. 2014;4:127.
28. Gahan PB, Stroun M. The virtosome-a novel cytosolic informative entity and intercellular messenger. Cell Biochem Funct. 2010;28:529–38.
29. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch R-D, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001;61:1659–65.
30. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci USA. 2005;102:16368–73.
31. García-Olmo DC, Ruiz-Piqueras R, García-Olmo D. Circulating nucleic acids in plasma and serum (CNAPS) and its relation to stem cells and cancer metastasis: state of the issue. Histol Histopathol. 2004;19:575–83.
32. Vogel TJ, DelloRusso C, Welcsh P, Shah CA, Goff BA, Garcia RL, et al. Angiogenic alterations associated with circulating neoplastic DNA in ovarian carcinoma. Transl Oncol. 2012;5:247–51.
33. García-Olmo D, García-Olmo DC, Ontañón J, Martinez E, Vallejo M. Tumor DNA circulating in the plasma might play a role in metastasis. The hypothesis of the genometastasis. Histol Histopathol. 1999;14:1159–64.
34. García-Olmo DCDDC, Domínguez C, García-Arranz M, Anker P, Stroun M, García-Verdugo JM. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. Cancer Res. 2010;70:560–7.
35. Trejo-Becerril C, Pérez-Cárdenas E, Taja-Chayeb L, Anker P, Herrera-Goepfert R, Medina-Velázquez LA, et al. Cancer progression mediated by horizontal gene transfer in an in vivo model. PLoS One. 2012;7:e52754.
36. Abdouh M, Zhou S, Arena V, Arena M, Lazaris A, Onerheim R, et al. Transfer of malignant trait to immortalized human cells following exposure to human cancer serum. J Exp Clin Cancer Res. 2014;33:86.
37. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HRH. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn. 2005;5:209–19.
38. Hohaus S, Giachelia M, Massini G, Mansueto G, Vannata B, Bozzoli V, et al. Cell-free circulating DNA in Hodgkin's and non-Hodgkin's lymphomas. Ann Oncol. 2009;20:1408–13.
39. Pinzani P, Salvianti F, Zaccara S, Massi D, De Giorgi V, Pazzagli M, et al. Circulating cell-free DNA in plasma of melanoma patients: qualitative and quantitative considerations. Clin Chim Acta. 2011;412:2141–5.
40. Mouliere F, Robert B, Peyrotte EA, Rio MD, Ychou M, Molina F, et al. High fragmentation characterizes tumour-derived circulating DNA. PLoS One. 2011;6(9):e23418.
41. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci. 1996;93:9821–6.
42. Wong IHN, Zhang J, Lai PBS, Lau WY, Lo YMD. Quantitative analysis of tumor-derived methylated p16INK4a sequences in plasma, serum, and blood cells of hepatocellular carcinoma patients. Clin Cancer Res. 2003;9:1047–52.
43. Chan KCA, Lai PBS, Mok TSK, Chan HLY, Ding C, Yeung SW, et al. Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. Clin Chem. 2008;54:1528–36.
44. Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, et al. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. J Clin Oncol. 2006;24:4262–9.
45. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci USA. 1999;96:9236–41.
46. Gevensleben H, Garcia-Murillas I, Graeser MK, Schiavon G, Osin P, Parton M, et al. Noninvasive detection of HER2 amplification with plasma DNA digital PCR. Clin Cancer Res. 2013;19:3276–84.
47. Taly V, Pekin D, Benhaim L, Kotsopoulos SK, Le Corre D, Li X, et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem. 2013;59:1722–31.
48. Leary RJ, Kinde I, Diehl F, Schmidt K, Clouser C, Duncan C, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med. 2010;2:20ra14.
49. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med. 2012;4:162ra154.

50. Newman AM, Bratman SV, To J, Wynne JF, Eclov NCW, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014;20:548–54.
51. Rothé F, Laes J-F, Lambrechts D, Smeets D, Vincent D, Maetens M, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann Oncol. 2014;25:1959–65.
52. Mohan S, Heitzer E, Ulz P, Lafer I, Lax S, Auer M, et al. Changes in colorectal carcinoma genomes under anti-EGFR therapy identified by whole-genome plasma DNA sequencing. PLoS Genet. 2014;10:e1004271.
53. Taniguchi K, Uchida J, Nishino K, Kumagai T, Okuyama T, Okami J, et al. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. Clin Cancer Res. 2011;17:7808–15.
54. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res. 2012;18:3462–9.
55. Nielsen PE, Egholm M. An introduction to peptide nucleic acid. Curr Issues Mol Biol. 1999;1:89–104.
56. Kim H-R, Lee SY, Hyun D-S, Lee MK, Lee H-K, Choi C-M, et al. Detection of EGFR mutations in circulating free DNA by PNA-mediated PCR clamping. J Exp Clin Cancer Res. 2013;32:50.
57. Däbritz J, Hänfler J, Preston R, Stieler J, Oettle H. Detection of Ki-ras mutations in tissue and plasma samples of patients with pancreatic cancer using PNA-mediated PCR clamping and hybridisation probes. Br J Cancer. 2005;92:405–12.
58. Sozzi G, Conte D, Leon M, Ciricione R, Roz L, Ratcliffe C, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. J Clin Oncol. 2003;21:3902–8.
59. Gal S, Fidler C, Lo YMD, Taylor M, Han C, Moore J, et al. Quantitation of circulating DNA in the serum of breast cancer patients by real-time PCR. Br J Cancer. 2004;90:1211–5.
60. Al-Shuneigat JM, Mahgoub SS, Huq F. Colorectal carcinoma: nucleosomes, carcinoembryonic antigen and ca 19-9 as apoptotic markers; a comparative study. J Biomed Sci. 2011;18:50.
61. Mussolin L, Burnelli R, Pillon M, Carraro E, Farruggia P, Todesco A, et al. Plasma cell-free DNA in paediatric lymphomas. J Cancer. 2013;4:323–9.
62. Gautschi O, Bigosch C, Huegli B, Jermann M, Marx A, Chassé E, et al. Circulating deoxyribonucleic acid as prognostic marker in non-small-cell lung cancer patients undergoing chemotherapy. J Clin Oncol. 2004;22:4157–64.
63. Tomita H, Ichikawa D, Ikoma D, Sai S, Tani N, Ikoma H, et al. Quantification of circulating plasma DNA fragments as tumor markers in patients with esophageal cancer. Anticancer Res. 2007;27:2737–41.
64. Trejo-Becerril C, Pérez-Cárdenas E, Treviño-Cuevas H, Taja-Chayeb L, García-López P, Segura-Pacheco B, et al. Circulating nucleosomes and response to chemotherapy: an in vitro, in vivo and clinical study on cervical cancer patients. Int J Cancer. 2003;104:663–8.
65. Heidary M, Auer M, Ulz P, Heitzer E, Petru E, Gasch C, et al. The dynamic range of circulating tumor DNA in metastatic breast cancer. Breast Cancer Res. 2014;16:421.
66. Shapiro B, Chakrabarty M, Cohn EM, Leon SA, Vegas L, Servi P. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer. 1983;51:2116–20.
67. Kamat AA, Baldwin M, Urbauer D, Dang D, Han LY, Godwin A, et al. Plasma cell-free DNA in ovarian cancer: an independent prognostic biomarker. Cancer. 2010;116:1918–25.
68. Yoon K-A, Park S, Lee SH, Kim JH, Lee JS. Comparison of circulating plasma DNA levels between lung cancer patients and healthy controls. J Mol Diagn. 2009;11:182–5.
69. Mouliere F, El Messaoudi S, Gongora C, Guedj A-S, Robert B, Del Rio M, et al. Circulating cell-free DNA from colorectal cancer patients may reveal high KRAS or BRAF mutation load. Transl Oncol. 2013;6:319–28.
70. Jiang P, Chan CWM, Chan KCA, Cheng SH, Wong J, Wong VW-S, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci. 2015;112:201500076.
71. Umetani N, Giuliano AE, Hiramatsu SH, Amersi F, Nakagawa T, Martino S, et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. J Clin Oncol. 2006;24:4270–6.
72. Dawson S-J, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin S-F, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;368:1199–209.
73. Douillard J-Y, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, et al. First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study. Br J Cancer. 2014;110:55–62.
74. Marcq M, Vallée A, Bizieux A, Denis MG. Detection of EGFR mutations in the plasma of patients with lung adenocarcinoma for real-time monitoring of therapeutic response to tyrosine kinase inhibitors? J Thorac Oncol. 2014;9:e49–50.
75. Kimura H, Suminoe M, Kasahara K, Sone T, Araya T, Tamori S, et al. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer. 2007;97:778–84.
76. Holdhoff M, Schmidt K, Donehower R, Diaz LA. Analysis of circulating tumor DNA to confirm somatic KRAS mutations. J Natl Cancer Inst. 2009;101:1284–5.
77. Elshimali YI, Khaddour H, Sarkissyan M, Wu Y, Vadgama JV. The clinical utilization of circulating cell free DNA (CCFDNA) in blood of cancer patients. Int J Mol Sci. 2013;14:18925–58.
78. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med. 1996;2:1035–7.
79. Chen XQ, Stroun M, Magnenat J-L, Nicod LP, Kurt A-M, Lyautey J, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med. 1996;2:1033–5.
80. Taback B, Fujiwara Y, Wang H-J, Foshag LJ, Morton DL, Hoon DSB. Prognostic significance of circulating microsatellite markers in the plasma of melanoma patients. Cancer Res. 2001;61:5723–6.
81. Fujiwara Y, Chi DDJ, Wang H, Keleman P, Morton DL, Turner R, et al. Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients. Cancer Res. 1999;59:1567–71.
82. Hamana K, Uzawa K, Ogawara K, Shiiba M, Bukawa H, Yokoe H, et al. Monitoring of circulating tumour-associated DNA as a prognostic tool for oral squamous cell carcinoma. Br J Cancer. 2005;92:2181–4.
83. Kakimoto Y, Yamamoto N, Shibahara T. Microsatellite analysis of serum DNA in patients with oral squamous cell carcinoma. Oncol Rep. 2008;20:1195–200.
84. Sunami E, Shinozaki M, Higano CS, Wollman R, Dorff TB, Tucker SJ, et al. Multimarker circulating DNA assay for assessing blood of prostate cancer patients. Clin Chem. 2009;55:559–67.
85. Hickey KP, Boyle KP, Jepps HM, Andrew AC, Buxton EJ, Burns PA. Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients. Br J Cancer. 1999;80:1803–8.

86. Baylin SB. DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol. 2005;2:S4–11.
87. Wong IHN, Zhang J, Lai PBS, Lau WY, Lo YMD. Quantitative analysis of tumor-derived methylated p16INK4a sequences in plasma, serum, and blood cells of hepatocellular carcinoma patients. Clin Cancer Res. 2003;9:1047–52.
88. Majchrzak-Celińska A, Paluszczak J, Kleszcz R, Magiera M, Barciszewska A-MM, Nowak S, et al. Detection of MGMT, RASSF1A, p15INK4B, and p14ARF promoter methylation in circulating tumor-derived DNA of central nervous system cancer patients. J Appl Genet. 2013;54:335–44.
89. Wong IHN, Dennis Lo YM, Zhang J, Liew C-T, Ng MHL, Wong N, et al. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res. 1999;59:71–3.
90. Muller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, et al. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res. 2003;63:7641–5.
91. Silva JM, Dominguez G, Villanueva MJ, Gonzalez R, Garcia JM, Corbacho C, et al. Aberrant DNA methylation of the p16INK4a gene in plasma DNA of breast cancer patients. Br J Cancer. 1999;80:1262–4.
92. An Q, Liu Y, Gao Y, Huang J, Fong X, Li L, et al. Detection of p16 hypermethylation in circulating plasma DNA of non-small cell lung cancer patients. Cancer Lett. 2002;188:109–14.
93. Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, et al. Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res. 2001;7:3135–8.
94. Nakayama H, Hibi K, Taguchi M, Takase T, Yamazaki T, Kasai Y, et al. Molecular detection of p16 promoter methylation in the serum of colorectal cancer patients. Cancer Lett. 2002;188:115–9.
95. Deligezer U, Yaman F, Erten N, Dalay N. Frequent copresence of methylated DNA and fragmented nucleosomal DNA in plasma of lymphoma patients. Clin Chim Acta. 2003;335:89–94.
96. Wong IHN, Lo YMD, Yeo W, Lau WY, Johnson PJ. Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. Clin Cancer Res. 2000;6:3516–21.
97. Wong T-S, Man MW-L, Lam AK-Y, Wei WI, Kwong Y-L, Yuen APW. The study of p16 and p15 gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR. Eur J Cancer. 2003;39:1881–7.
98. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum dna from non-small cell lung cancer patients. Cancer Res. 1999;59:67–70.
99. Rosas SLB, Koch W, da Costa Carvalho MDG, Wu L, Califano J, Westra W, et al. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res. 2001;61(3):939–42.
100. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res. 2000;60:892–5.
101. Usadel H, Brabender J, Danenberg KD, Jeronimo C, Harden S, Engles J, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma dna of patients with lung cancer. Cancer Res. 2002;62:371–5.
102. Avraham A, Uhlmann R, Shperber A, Birnbaum M, Sandbank J, Sella A, et al. Serum DNA methylation for monitoring response to neoadjuvant chemotherapy in breast cancer patients. Int J Cancer. 2012;131:E1166–72.
103. Mori T, O'Day SJ, Umetani N, Martinez SR, Kitago M, Koyanagi K, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol. 2005;23:9351–8.
104. Müller HM, Widschwendter A, Fiegl H, Marth C, Widschwendter M. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res. 2003;63(22):7641–5.
105. Van der Auwera I, Elst HJ, Van Laere SJ, Maes H, Huget P, van Dam P, et al. The presence of circulating total DNA and methylated genes is associated with circulating tumour cells in blood from breast cancer patients. Br J Cancer. 2009;100:1277–86.
106. Matuschek C, Bölke E, Lammering G, Gerber PA, Peiper M, Budach W, et al. Methylated APC and GSTP1 genes in serum DNA correlate with the presence of circulating blood tumor cells and are associated with a more aggressive and advanced breast cancer disease. Eur J Med Res. 2010;15:277–86.
107. Skvortsova TE, Rykova EY, Tamkovich SN, Bryzgunova OE, Starikov AV, Kuznetsova NP, et al. Cell-free and cell-bound circulating DNA in breast tumours: DNA quantification and analysis of tumour-related gene methylation. Br J Cancer. 2006;94:1492–5.
108. Zhou L, Liu J, Luo F. Serum tumor markers for detection of hepatocellular carcinoma. World J Gastroenterol. 2006;12:1175–81.
109. Philipp AB, Nagel D, Stieber P, Lamerz R, Thalhammer I, Herbst A, et al. Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer. BMC Cancer. 2014;14:245.
110. Wallner M, Herbst A, Behrens A, Crispin A, Stieber P, Göke B, et al. Methylation of serum DNA is an independent prognostic marker in colorectal cancer. Clin Cancer Res. 2006;12:7347–52.
111. Ostrow KL, Hoque MO, Loyo M, Brait M, Greenberg A, Siegfried JM, et al. Molecular analysis of plasma DNA for the early detection of lung cancer by quantitative methylation-specific PCR. Clin Cancer Res. 2010;16:3463–72.
112. Ling Z-QQ, Lv P, Lu X-XX, Yu J-LL, Han J, Ying LSS, et al. Circulating methylated XAF1 DNA indicates poor prognosis for gastric cancer. PLoS One. 2013;8:67195.
113. Raja UM, Gopal G, Rajkumar T. Intragenic DNA methylation concomitant with repression of ATP4B and ATP4A gene expression in gastric cancer is a potential serum biomarker. Asian Pac J Cancer Prev. 2012;13:5563–8.