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Monitoring tumor-derived cell-free DNA in patients with solid tumors: Clinical perspectives and research opportunities

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Abstract

Circulating cell-free DNA represents a non-invasive biomarker, as it can be isolated from human plasma, serum and other body fluids. Circulating tumor DNA shed from primary and metastatic cancers may allow the non-invasive analysis of the evolution of tumor genomes during treatment and disease progression through 'liquid biopsies'. The serial monitoring of tumor genotypes, which are instable and prone to changes under selection pressure, is becoming increasingly possible. The "liquid biopsy" provide novel biological insights into the process of metastasis and may elucidate signaling pathways involved in cell invasiveness and metastatic competence.

This review will focus on the clinical utility of circulating cell free DNA in main solid tumors, including genetic and epigenetic alterations that can be detected.

Keywords

- Cell free DNA;
- Plasma/serum DNA;
- Circulating nucleic acids;
- Breast cancer;
- Lung cancer;
- Colorectal cancer;
- Prostate cancer;
- Ovarian cancer

Introduction

Improvement in the clinical outcome of many cancer types is likely to be achieved by giving patients a drug tailored to the genetic makeup of their tumor. The recent surge in high-throughput sequencing of cancer genomes is delivering more accurate tumor genome information, and it can be anticipated that more

predictive biomarkers will be identified and that patients will increasingly be treated by focusing on the genetic architecture of their particular tumor rather than on the tumor's location or histological features. Circulating cell-free DNA (cf-DNA) represents a non-invasive biomarker, as it can be isolated from human plasma, serum and other body fluids [1]. Cf-DNA offer a unique opportunity for serially monitoring tumor genomes in a non-invasive manner. As cf-DNAs is a potential surrogate for the tumor itself, it is often referred to as 'liquid biopsy'.

In 1940 Mandel and Métais observed, for the first time, the presence of cell-free nucleic acid in the blood of healthy individuals [2].

Thirty years later, Leon et al. reported elevated levels of cf-DNA in the circulation of cancer patients in comparison with healthy individuals [3] and the data were confirmed in several studies [4], [5] and [6].

Subsequently, several studies reported a variety of alterations in cf-DNA such as mutations in oncogenes and tumor suppressor genes [7], microsatellite variances [8], and promoter hypermethylation [9].

The levels of cf-DNA might also reflect physiological and pathological processes that are not tumor-specific. Increased levels may be found in patients with benign lesions, inflammatory diseases and tissue trauma [10], which makes it difficult to evaluate the extent to which cf-DNA in the circulation of a patient is cancer specific.

Although the data on cf-DNA are extensive, the possible usefulness of this marker in the clinical setting remains controversial and, presently, the evaluation of cf-DNA is not yet approved in any clinical guideline.

In our review we will focus on the findings on cf-DNA in breast, lung, colorectal, ovarian and prostate cancers and discuss its relevance and potential use as diagnostic and prognostic marker.

Biology of circulating cell free DNA

The source of cf-DNA is still uncertain. A proportion seems to derive from nucleated blood cells; wild-type DNA has been detected in the plasma of cancer patients as well as in that of healthy controls. In cancer patients a considerable proportion of plasma DNA originates from tumor cells [11]. The apoptosis and necrosis of cancer cells in the tumor microenvironment are the possible explanations for the release of the nucleic acids into the blood.

Macrophages usually engulf necrotic and apoptotic cells and then they release the digested DNA into the tissue environment [11]. Another hypothesis is that cf-DNA is due to the lyses of circulating cancer cells or micro metastases shed by tumor [11]. It was also hypothesized that the tumor actively releases DNA into the bloodstream [11].

The amount of cf-DNA that derives from tumor cells changes owing to the size and the state of the tumor. The proportion of cf-DNA is also conditioned by clearance, degradation and other physiological filtering events of the blood and lymphatic circulation[12]. However the concentration of cf-DNA in serum of cancer patients is about 4 times that of healthy controls [13]. The size of the DNA released from dead cancer cells varies between small fragments of 70–200 base pairs and large fragments of about 21 kilobases and it is longer than that of non neoplastic DNA [14].

Breast cancer

Quantitative alterations of circulating DNA

Some quantitative studies reported increased concentrations of circulating DNA in patients with breast cancer when compared to healthy individuals [15], [16] and [17]. Frattini et al. demonstrated that the levels of cf-DNA present in cancer patients constitute a stable parameter over the time and its variations may be correlated with clinical outcome [15]. Large individual variations in DNA quantity have been observed among different studies. A comprehensive review reported that the concentration of DNA in the bloodstream of patients with breast cancer (BC) varied from 153 to 549 ng/ml in serum and from 122 to 462 ng/ml in plasma, whereas that of healthy controls ranged between 63–318 and 3–63 ng/ml, respectively [10].

A study by Kohler et al. showed significantly higher levels of cf-DNA in patients with BC in comparison to the patients with benign breast tumors and healthy individuals. This quantitative approach could distinguish between cancer patients and healthy controls with a sensitivity and specificity of 81% and 69%, respectively [16].

Catarino et al. quantified circulating DNA using real-time PCR (polymerase chain reaction) and showed that the cf-DNA levels were higher before breast surgery respect to post-operative time. This observation proved that cf-DNA may be a useful tool for monitoring disease. In addition, elevated levels of cf-DNA were found to be associated with tumor size, lymph node involvement, histopathological grade, and clinical staging [17]. These results were in accordance with previous observations in which the cf-DNA of BC patients also correlated with stage, lymph node metastasis and tumor size [4] and [18].

Qualitative alterations of circulating DNA

Several studies reported that a qualitative analysis of specific molecular alterations in isolated DNA from tumoral tissue may mirror cf-DNA [19]. The presence of specific mutations helps to differentiate circulating tumor DNA (ct-DNA) from normal cf-DNA. These somatic mutations, commonly single base pair substitutions, are present only in the genomes of cancer cells or pre-cancerous cells and are never present in the DNA of normal cells of the same individual. This juxtaposition confers to ct-DNA elevated specificity as a biomarker.

Four studies, for a total of 371 patients with breast cancer, analyzed the mutations of the tumor suppressor gene *TP53* [20], [21], [22] and [23].

Great variations in incidence were described in the 4 different studies, ranging from 11% to 73% and 5% to 43% for tumors and circulating DNA, respectively. These great variations should be considered as the consequence of the different methodologies used.

Also the *PIK3CA* mutations were detected in the plasma in up to 30% of patients with advanced breast cancer [24], [25] and [26]. In the study of Board and colleagues [24], the *PIK3CA* mutations were detected in cf-DNA of 13/46 (28%) patients with metastatic breast cancer but were not detected in cf-DNA of patients with operable breast cancer. The concordance was 95% for *PIK3CA* mutations found in cf-DNA compared to those found in tumor DNA. These results clearly demonstrated that the detection of cf-DNA mutations is lower in the early stage disease compared to more advanced disease and demonstrated the feasibility of detection of *PIK3CA* mutation in cf-DNA.

A more recent study showed how the analysis of ct-DNA can be integrated in the clinical management of BC patients [27].

Dawson et al. [27] compared the radiographic imaging of tumors with the assay of ct-DNA, CA 15.3 and circulating tumor cells in 30 patients with advanced breast cancer during chemotherapy administration. Ct-DNA was identified in 29 of the 30 women (97%) in whom somatic genomic alterations of *TP53* and *PIK3A* were detected. This assay proved to have a higher sensitivity for detecting presence of metastatic disease when compared with assays of CA 15-3 or circulating tumor cells. Furthermore, the researchers found that measurement of levels of ct-DNA corresponds both with treatment response and survival: those who survived longer had lower levels of ct-DNA compared with those who survived a shorter period of time. These initial studies involved patients with high tumor burden and it remains to be evaluated if similar sensitivity can be achieved in patients with localized disease.

Additional genetic alterations that are detectable on cf-DNA and are used as biomarkers in cancer include the integrity of non-coding genomic DNA repeat sequences such as *ALU* and *LINE1*.

The *ALU* and *LINE1* sequences are involved in various physiological events such as DNA repair, transcription, epigenetic and transposon-based activity [28] and many studies showed their potential prognostic and diagnostic utility [29] and [30]. The assays are based on the observation that common DNA repeat sequences are preferentially released by tumor cells that are undergoing non apoptotic or necrotic cell death, and these fragments can be between 200 and 400 bp in size [12]. The integrity of cf-DNA *ALU* sequences in blood has been shown to be sensitive for the assessment of the early stage of BC progression, including micrometastasis [30].

Also the microsatellite instability [MSI] and loss of heterozygosity [LOH] of cf-DNA were observed to have potential application in the management of patients with BC [6],[8] and [31].

A recent study [31] showed that the detection of DNA losses of the tumor suppressor genes *TIG1*, *PTEN*, *cyclin D2*, *RB1*, and *BRCA1* on cf-DNA was associated with a more aggressive biology of BC. In particular, the detected *cyclin D2* loss was an indicator of an unfavorable prognosis. Thus, the improved detection of cf-DNA might provide clinically relevant information on the variable biology of BC. In addition, LOH was not detected in the healthy subjects whereas it was observable in patients with carcinoma in situ [6] indicating the potential application of this analysis in the screening proceedings. Recently it has been observed that epigenetic changes are a constant alterations in cancer cells and they have a substantial effect on early carcinogenesis and progression [32]. The most examined epigenetic modification is that of DNA methylation.

A small set of genes (*APC*, *RASSF1A*, *DAP kinase*) were found hypermethylated in the cf-DNA of patients with in situ carcinoma and with benign lesions [33], suggesting that this approach may be of particular interest in view of early molecular diagnosis. In addition it was seen that hypermethylation of *RASSF1A* and *APC* detected in serum DNA of BC patients was associated with a worse outcome [34]. It was seen, also, that the combined assessment of large amounts of free circulating total/methylated DNA and circulating tumor cells was predictive for tumor progression [35]. Other studies suggested that the assessment of abnormal methylation in the serum of BC patients may be used for monitoring the efficacy of neoadjuvant [36] and adjuvant [37] and [38] treatment. Therefore, the detection of methylated cf-DNA represents a promising approach for risk assessment in cancer patients.

Lung cancer

Quantitative alterations of circulating DNA

The quantitative analysis of cf-DNA as tool for the lung cancer monitoring was investigated in many studies. Significantly higher DNA concentrations were detected in the serum/plasma of lung cancer patients compared with healthy controls or patients with benign diseases [39] and [40].

In addition higher DNA levels have been reported in the serum of patients with metastatic disease than in patients with non metastatic disease, and it was seen that the cf-DNA levels decreased in 75% of the patients after therapy [3].

Sozzi et al. reported that the cf-DNA concentration was significantly lower in plasma of patients during the follow up than before surgery and was comparable to the concentration measured in the control group, suggesting that quantification of plasma DNA might represent a new approach to monitor surgical procedures or to assess the efficacy of chemo/radiotherapy [40].

Plasma DNA concentrations do not uniformly correlate with disease stage and histological subtypes [41]. Conflicting data exist regarding to the correlation of cf-DNA with survival. Some authors reported no correlation between plasma DNA concentrations and relapse-free or overall survival [40] and [41], whereas other authors reported an association of plasma DNA with survival, lactate dehydrogenase [39] and [42], and NSE [42] for a mixed group of SCLC and NSCLC patients [42], and for NSCLC patients only [39].

Qualitative alterations of circulating DNA

Several oncogenes are involved in lung carcinogenesis, such as *Ras*, *c-myc*, tyrosine-kinase receptors (*EGFR*) and *c-erbB2* (*HER2/neu*).

Ramirez et al. reported *K-ras* mutations in 24% of 50 resected NSCLC patients [43] and Kimura et al. detected mutations in 20% of cases [44], whereas Bearzatto et al. did not find *K-ras* mutations in serum [45].

Camps et al. demonstrated that there were no significant differences between the NSCLC patients with *K-ras* mutations in the serum and those with wild-type genotype with respect to baseline patient characteristics, response rates, progression-free survival, or overall survival [46]. Instead two other studies found an association between the presence of mutant *K-ras* in serum/plasma of NSCLC patients and survival [43] and [44]. In a more large study the plasma DNA of 180 lung patients was analyzed and it was found a significant correlation between the presence of *K-ras* mutations and poor prognosis [47].

The examination of *EGFR* mutations is essential to determine an appropriate lung cancer treatment strategy. Several clinical trials endorsed that the response rate to *EGFR*-TKI is approximately 70% in patients with *EGFR* activating/sensitive mutations, such as exon 19 deletions or L858R mutation [48].

Many studies have focused on detection of mutations in plasma or serum, to avoid the difficulty to obtain tumor specimens.

Kimura et al. analyzed the *EGFR* mutation status in tumor and plasma of 42 patients treated with gefitinib. *EGFR* mutations were detected in the tumor samples of eight patients and in the serum samples of seven patients highlighting that *EGFR* mutation status in serum DNA was the same as in tumor samples in almost every patient. In addition, they demonstrated a correlation between the presence of

the *EGFR* mutations in plasma DNA and the objective responses to gefitinib and as well as a trend towards increased overall survival in patients treated with gefitinib [49].

The EURTAC trial [50] reported, for patients with *EGFR* mutations detected in serum, an hazard ratio (HR) of 0.25 in favor of erlotinib, which was similar to results from IPASS study (HR 0.29 in favor of gefitinib) [51].

Moreover, in the EURTAC trial, the subgroup analysis of *EGFR* mutations in cf-DNA showed that the presence of *EGFR* mutations was an independent prognostic marker for progression free survival (PFS) (HR 0.43, 95% CI 0.26–0.73; $p = 0.002$). The PFS for patients with mutations detected in serum was 10.7 months (95% CI 6.8–15.5) in the erlotinib group compared with 4.2 months (3.2–6.0) in the standard chemotherapy group (HR 0.25, 95% CI 0.12–0.54; $p = 0.0002$). PFS for patients in whom mutations were not detected was 12.6 months (95% CI 8.3–not assessable) in the erlotinib group compared with 6.0 months (4.9–9.0) in the standard chemotherapy group (HR 0.29, 95% CI 0.13–0.63; $p = 0.0010$) [50].

Another study demonstrated that the plasma levels of the mutant sequences of *EGFR* correlated with the clinical response and that a decreased concentration was detected in all patients with partial or complete clinical remission, whereas persistence of mutation was observed in the patient with cancer progression [52].

These studies suggest that the detection of *EGFR* and other mutations in cf-DNA could be used to monitoring response treatment and to detect the presence of new acquired mutations in plasma or serum. In 50% of lung cancer patients, acquired resistance to gefitinib or erlotinib, develops through the emergence of *EGFR* T790M variants [53]. The mutation at residue 790 increases the affinity of *EGFR* for ATP and so out-competes binding of the inhibitors. These results have been initially obtained examining biopsies from patients who relapsed upon anti-*EGFR* and were later confirmed through the analyses of plasma, providing the first example that resistance to targeted therapies of solid tumors can be detected non-invasively in the blood of patients [54].

It was observed also that the MSI, the LOH and the epigenetic alterations of cf-DNA may have a potential application in the management of patients with lung cancer [40], [45], [55], [56] and [57].

Colorectal cancer

Quantitative alterations of circulating DNA

The detection of circulating DNA has strengthened its role as an additional tool for the management of patients with colorectal cancer (CRC).

A study by Frattini et al. suggested that the quantification of plasma tumor DNA might be useful for monitoring patients with colorectal cancer and, prospectively, for recognizing high-risk individuals [58]. The data of this study showed that the plasma tumor DNA levels were significantly higher in patients with CRC, they decreased progressively in the follow up period in tumor-free patients and instead they increased in patients with recurrence or metastasis [58]. These results are in line with the findings of another study which demonstrated that serum DNA levels were significantly increased in stage IV CRC patients and they fluctuated during chemotherapy [59].

A study performed in a cohort of patients with CRC undergoing resection with curative intent showed that it is possible to define a unique set of molecular probes for each patient. These probes can then be

exploited to quantify tumoral DNA after surgery. The patients were subsequently followed over the course of 2–5 years. In this study, ct-DNA was shown to be sufficiently sensitive to detect minimal residual disease after surgical resection [60].

Other studies reported higher levels of circulating DNA in CRC patients than in healthy individuals, but a correlation was not demonstrated between the levels of circulating DNA and the size and site of the tumor and the clinical course of disease [61] and [62].

Another study compared the sensitivity and specificity of cf-DNA with that of conventional serum marker carcinoembryonic antigen (CEA) and it was showed that cf-DNA, when used in combination with CEA, represents a potentially useful tool for the diagnosis of early stage CRC [63].

Qualitative alterations of circulating DNA

Although anti-EGFR therapy has established efficacy in metastatic colorectal cancer (MCRC), only 10–20% of unselected patients respond [64]. This is partly due to *KRAS* mutation, which is currently assessed in the primary tumor. Many studies assessed mutation status in circulating tumor DNA, with the aim to improve patient selection [65], [66], [67] and [68].

In the study published by Bazan et al., it was shown that the preoperative detection of mutant *KRAS* and *TP53* in the serum of CRC patients undergoing resective surgery, was predictive of disease recurrence [65]. In another study it was detected the amount of circulating tumor DNA in 18 MCRC patients who underwent surgical resection of their metastases and it was observed that the detection of circulating mutant DNA after surgery was highly predictive of disease recurrence [66]. These results were agree with those of a previous study in which the probability to have a curative surgical resection has been shown to be lower in MCRC patients with measurable *KRAS* mutation in their serum [67].

Lefebure et al. detected, in the serum of unresectable MCRC, *KRAS* mutation and *RASSF2A* methylation and they observed that the presence of circulating mutant DNA was predictive of clinical outcome [68]. In a recent study was demonstrated the clinical utility of multiplex digital PCR to screen for multiple *KRAS* mutations in the plasma samples of MCRC patients [69] suggesting that the “liquid biopsy” is a feasible alternative to a solid tissue biopsy for identifying specific mutations.

The principal limitations of therapies that target the extracellular domain of *EGFR* is the acquisition of secondary drug resistance. In a recent study was investigated whether the detection of *KRAS* mutant alleles, in the plasma of patients become refractory to anti-EGFR therapies, may allow the early identification of individuals at risk of develop drug resistance before radiographic documentation of disease progression [70].

It was found that *KRAS* mutant alleles were detectable in the blood of cetuximab treated patients as early as 10 months before the documentation of disease progression by radiologic assessment. Of relevance, the discovery of the mechanisms of resistance to the anti EGFR antibodies therapies in CRC was simultaneously accomplished in tissue and liquid biopsy. Another noteworthy aspect of this study was the emergence of multiple different resistance mutations in the same patient [70].

Overall these results indicate that the emergence of *KRAS* mutant clones can be detected months before radiographic progression and could allow an early initiation of combination therapies that may delay or prevent disease progression.

DNA hypermethylation in serum of colorectal cancer patients has been reported to have prognostic and predictive value [61], [71], [72] and [73] and together with the assessment of DNA integrity [74] represents another promising tool for risk assessment.

Prostate cancer

Quantitative alterations of circulating DNA

We need specific, sensitive and non invasive biomarkers for prostate cancer (PCA) diagnosis that could be used in addition to prostate-specific antigen (PSA) serial evaluations. The measurement of ct-DNA as a marker of tumor dynamics over conventional PSA or even imaging studies has been actively explored.

Two studies failed to demonstrate significant differences of ct-DNA between patients with PCA and non malignant prostate disease [75] and [76]. In the first study [75] the failure could be due to the use of a less sensitive fluorometric assay, whereas other studies used a real time PCR and detected higher DNA levels in PCA patients than in non malignant prostate disease patients [77] and [78]. Cf-DNA levels were correlated with pathological stage [78], Gleason score, surgical margin status and extraprostatic extension [79]. In addition it was showed that cf-DNA increased with diagnosis of metastasis and was predictive of survival in patients with metastatic prostate cancer [75],[79] and [80].

Qualitative alterations of circulating DNA

One of the earliest genetic alterations during prostate carcinogenesis seems to be *GSTP1* CpG island hypermethylation. A recent meta-analysis of 22 manuscripts indicated good specificity (89%) but modest sensitivity (52%) of *GSTP1* differential methylation for PCA screening [81]. The detection of hypermethylated DNA is also an adverse prognostic marker: *RASSF1A*, *RARB2*, and *GSTP1* hypermethylation were correlated with the Gleason score and the serum PSA [82]. *RARB2* and *GSTP1* methylation were also correlated with the AJCC stage [82]. *GSTP1* hypermethylation was the strongest predictor of PSA recurrence following radical prostatectomy [83] and it was correlated with the Gleason score and the extent of metastasis in patients with hormone-refractory PCA [84].

Also the analysis of the fragmentation patterns of cf-DNA provide useful information in the management of prostate cancer. It was seen that the DNA integrity was higher in PCA patients than in controls [85]. Ellinger et al. demonstrated that the DNA integrity was a predictor of PSA recurrence following radical prostatectomy and that the DNA integrity may allow to recognize PCA patients and benign prostate hyperplasia patients with a specificity of 81% and a sensitivity of 68% [77].

Ovarian cancer

Quantitative alterations of circulating DNA

Ovarian cancer is the fifth leading cause of cancer death [86] in women.

The cf-DNA has been investigated as novel biomarker for diagnosis, prediction and monitoring of therapeutic response in ovarian cancer.

Kamat et al. showed that the levels of cf-DNA were elevated in patients with invasive epithelial ovarian cancer compared to the controls emphasizing the potential role of cf-DNA as an additional non-invasive technique to identify women with malignant ovarian disease [87]. These findings were not consistent with a subsequent study showing that there was no statistical difference in the levels of cf-DNA between patients with ovarian cancer and patients with benign ovarian tumors [88]. One explanation for these results is the elevation of cf-DNA by other causes, including variation in the source, in fact cf-DNA in serum is reported to be higher than in plasma.

In another study it was demonstrated also the utility of cf-DNA as surrogate biomarker of therapeutic response, using an orthotopic ovarian cancer model [89]. It was seen that cf-DNA correlated with tumor burden and the levels declined with chemotherapy.

Kamat et al. investigated also the role of pre-operative total plasma cf-DNA levels in predicting clinical outcome in patients with ovarian cancer [90]. The cf-DNA levels were significantly higher in patients with invasive cancer compared with women with benign ovarian tumors and controls and high cf-DNA levels correlated with aggressive phenotypic features such as high stage and high grade. In addition it was seen that cf-DNA >22,000 GE/ml was an independent predictor of poor outcome in patients with ovarian cancer, and was superior to CA 125 in predicting mortality [90]. Also another study reported a significant difference between the presence of cf-DNA in patients with high grade serous cancer compared with other histological subtypes [91].

Wimberger et al. observed a significant relationship between residual tumor load of >1 cm after primary surgery and serum DNA levels, and both parameters were associated with a higher risk of relapse and poorer overall survival [92].

Qualitative alterations of circulating DNA

As the measurement of cf-DNA levels has some limitations as screening marker for ovarian cancer, in last years the research has focused on findings of specific alterations in cf-DNA, such as methylation or microsatellite alterations and mutation of tumor suppressor genes.

In early detection of ovarian cancer, Liggett et al. found that methylation of *RASSF1A* and *PGR-PROX* in serum may be a useful biomarker to differentiate between benign and malignant ovarian tumors with a specificity of 73.3% and a sensitivity of 80.0% [93]. Another study showed that hypermethylation of at least one of six gene promoters, including *RASSF1A*, *BRCA1*, *APC*, *DAPK* and *CDKN2A*, was detected in the serum of early-stage ovarian cancer patients with 82% sensitivity; in contrast it was not reported hypermethylation in non-neoplastic tissue, peritoneal fluid, or serum from 40 control women (100% specificity) [94]. Abnormal methylation of the *HMLH1* promoter has been demonstrated to correlate with poor survival after chemotherapy [95] providing the possibility to identify those patients who relapse after standard chemotherapy and who would be suitable for novel epigenetic therapies.

In a small study, tumor specific mutated *p53* DNA levels were detected in the peritoneal fluid of 28 of 30 (93%) women with intraperitoneal ovarian cancer, including all 6 cases with non-malignant cells identified on cytopathology [96]. Therefore the identification of cf-DNA in peritoneal fluid compares favorably to cytopathologic evaluation, and warrants further investigation as a diagnostic marker.

In ovarian cancer patients were investigated also the MSI and the DNA integrity with promising results [97] and [98].

Conclusion and perspectives

The detection of cf-DNA provides new opportunities for management of cancer patients adding a new useful tool for diagnosis, staging and prognosis. It offers a new type of very specific biomarker that allow to identify the mutations accumulated from each tumor and to monitor the tumor burden and the response to treatment using a minimally invasive blood analyses (Table 1).

Table 1.

Detection of cf-DNA and its alterations in patients with solid tumors.

Cf-DNA alterations	Molecular alterations				
	Breast	Lung	Colorectal	Prostate	Ovary
<i>Mutation</i>	TP53 (28–31,37) PIK3CA (32–34,37)	RAS (63–67) EGFR (72–77)	RAS, TP53, APC (10,84,90,92–94)	–	TP53 (127)
<i>DNA integrity</i>	Serum DNA integrity (40,41)	–	Serum DNA integrity (100)	Serum DNA integrity (102,115)	Serum DNA integrity (129)
<i>Microsatellite alterations</i>	LOH and MSI (9,11,42,43)	LOH and MSI (8, 78)	–	LOH and MSI (110,112)	LOH (128)
<i>Methylation</i>	RASSF1A, APC, DAPK, ESR1, BRCA1, MGMT, GSTP1, Stratifin, MDR1, HSD17B4, HIC1, NEUROD1 (48–53)	P16 (65, 79) 14-3-3sigma (80)	SEPT9, ALX4, HLTF, HPP1 (84, 95–99)	GSTP1, RASSF1A, RARB2, AR (111–114)	RASSF1A, PGR PROX, BRCA1, APC, DAPK, CDKN2A, HMLH1 (124, 126)

LOH, loss of heterozygosity; MSI, microsatellite instability.

[Table options](#)

In addition cf-DNA may have particular utility in the identification of mutations associated with acquired drug resistance in advanced cancers allowing to avoid repeat biopsies and to provide an evaluation of clonal genomic evolution associated with treatment response and resistance [70] and [99].

There are, however, several questions to be answered. One crucial factor in evaluating cf-DNA is the standardization of assays and the definition of the optimal sampling specimen (serum or plasma) to obtain data more consistent and comparative between different laboratories. Actually there are different procedures both in the pre-analytical phases (blood collection, processing, storage, baseline of patients), in DNA extraction, in quantification and analysis after the DNA extraction. These variables are critical and need to be standardized for consensus analysis and reporting. Despite these technical limitations, “liquid biopsy” may provide a unique opportunity in the field of clinical cancer research and have been already embedded in the design of several clinical trials (Table 2). Correlation of qualitative and quantitative

analysis of circulating DNA with pathological response to neoadjuvant treatment in solid tumors may anticipate potential resistance to cytotoxic and biological agents during preoperative therapy. In the era of next generation sequencing detection of specific DNA mutations predicting response or resistance to targeted agents may anticipate switch to non cross-resistant therapies. The identification of circulating DNA in patients with breast cancer receiving neoadjuvant chemotherapy may predict pathological residual disease that correlate with poor prognosis in triple negative and HER2 positive breast cancer. Another opportunity to explore the role of cf-DNA is to study the “tumor dormancy” phenomenon. Analysis of cf-DNA in patients in follow up for previous diagnosis of breast cancer has provided some hints regarding tumor dormancy. Specifically identification of cf-DNA might be employed to monitor patients with breast cancer without clinically detectable disease [100] comparing the genomic profiles of germline DNA, cf-DNA and tumor DNA as defined by single nucleotide polymorphism arrays. It is essential to highlight that plasma cf-DNA and primary tumor tissue showed some similarities regarding specific tumor copy-number variations as observed by DNA amplification at several chromosome arms [100]. The biological significance of cf-DNA in patients with no evidence of clinical disease is presently unclear; further studies are required to determine whether this approach is sufficiently sensitive as a screening tool or for identification of early relapse. More importantly, the process of identifying specific DNA mutations for each patient’s cancer is a laborious process that is currently too time-intensive and costly for more widespread use. Future development will have to provide a cost effective analysis mainly identifying the genes known to be recurrently mutated in each tumor. Therefore, developing standardized methodologies for cf-DNA analyses and validation in large prospective clinical studies is mandatory to implement the ‘liquid biopsy’ approach in the clinical management of cancer patients.

Table 2.

Ongoing clinical trials that study cf-DNA in solid tumors with therapeutic intervention.

Clinical trial	Status	Therapeutic intervention	Setting
NCT00899548 [®]	Recruiting	Predict response after systemic therapy	MBC
NCT01198743 [®]	Recruiting	Validate prognostic value of cf-DNA	Stage II–III CRC
NCT00977457 [®]	Recruiting	Predict recurrence	Prostate cancer undergoing surgery
NCT01617915 [®]	Recruiting	Correlate cf-DNA with response to neoadjuvant CT	BC candidate to neoadjuvant CT
NCT01776684 [®]	Recruiting	Evaluation of EGFR TKI resistance mechanism	NSCLC
NCT01836640 [®]	Not yet recruiting	Evaluate cf-DNA as a surrogate for tumor biopsy to identify tumor genetic alterations	MBC

CT, chemotherapy; CRC, colorectal cancers; cf-DNA, circulating cell-free DNA; MBC, metastatic breast cancer; NSCLC, non small cell lung cancer; BC, breast cancer; EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor.

Conflict of interest

All of the authors declare no conflicts of interest.

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