HHS Public Access

Author manuscript

Cancer Genet. Author manuscript; available in PMC 2019 June 28.

Published in final edited form as:

Cancer Genet. 2018 December ; 228-229: 169–179. doi:10.1016/j.cancergen.2018.02.005.

Circulating cell-free DNA for non-invasive cancer management

Caitlin M. Stewart[a,b] and **Dana W.Y. Tsui**[a,b,*]

[a]Marie-José and Henry R. Kravis Center for Molecular Oncology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, Box 20, New York, NY 10065, USA;

[b]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Abstract

Cell-free DNA (cfDNA) was first identified in human plasma in 1948 and is thought to be released from cells throughout the body into the circulatory system. In cancer, a portion of the cfDNA originates from tumour cells, referred to as circulating-tumour DNA (ctDNA), and can contain mutations corresponding to the patient's tumour, for instance specific TP53 alleles. Profiling of cfDNA has recently become an area of increasing clinical relevance in oncology, in particular due to advances in the sensitivity of molecular biology techniques and development of next generation sequencing technologies, as this allows tumour mutations to be identified and tracked non-invasively. This has opened up new possibilities for monitoring tumour evolution and acquisition of resistance, as well as for guiding treatment decisions when tumour biopsy tissue is insufficient or unavailable. In this review, we will discuss the biology of cell-free nucleic acids, methods of analysis, and the potential clinical uses of these techniques, as well as the on-going clinical development of ctDNA assays.

Keywords

Molecular diagnostics; Liquid biopsy; Molecular pathology; Sequencing; Cell-free DNA; Cancer

Biology of cell-free DNA

cfDNA is believed to be released during normal cell functions, such as secretion and export in exosomes, as well as during cell death programs, such as apoptosis and necrosis [1,2]. It can be found in plasma [3] as well as other body fluids such as urine [4,5] , cerebral spinal fluid (CSF) [6] , pleural fluid [7] , and saliva [8,9] , among others. Once outside of the cell,

*Corresponding author at: Marie-José and Henry R. Kravis Center for Molecular Oncology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, Box 20, New York, NY 10065, USA. tsuiw@mskcc.org.

Author contribution statement

CMS wrote the initial draft, with contributions and guidance from DWYT. Both authors approved the final version.

Conflict of interest statement

DWYT is a former consultant of Inivata Ltd. DWYT is a contributor to patent on cell-free DNA detection methodologies and may receive royalties related to the licenses of those patents to Inivata Ltd, the terms of these royalties are managed by Cancer Research Technology and Cambridge Enterprise. DWYT has received travel sponsorship and honorarium from AstraZeneca. CMS has no conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2018.02.005.

cfDNA is steadily degraded by nucleases [10–12] , possibly with help from macrophages [13] , and excreted in urine via the renal system [14–17]. The half-life of cfDNA is very short according to studies done in prenatal medicine, between 16 min–2.5 h in plasma [18–20] , therefore it is often highly damaged and degraded. Despite this, the fast turnover of cfDNA can be useful for real-time tracking of genetic and genomic changes.

Multiple studies have demonstrated that the majority of cfDNAs are short molecules around 167 bp, although longer fragments also exist [21].167 bp corresponds approximately to the length of DNA wrapped around a histone, indicating cfDNA is likely associated with nucleosomes [22–24]. These fragments are believed to be released from cells in exosomes and extracellular vesicles, as well as during apoptosis [1]. Dying cells and tumours undergoing cell necrosis release additional longer fragments, up to 10 kb [21]. The majority of cfDNA in the blood originates from hematopoietic cells [25]; however tissue-specific methylation signatures of the cfDNA fragments has also identified fragments from many other organs [24–26].

Circulating tumour DNA

As cfDNA originates from many organs, ctDNA released from tumours within these organs can also be found within cfDNA samples. Data from xenograft experiments has shown that ctDNA is slightly shorter than cfDNA [27,28], approximately 134–144 bp [29,30]. Although the reason behind this shortening is not known, it is possible that this is the size of the DNA wrapped around a nucleosome without the linker DNA that connects histones or that increased time in circulation resulted in degradation. Interestingly, foetal DNA and cfDNA originating from transplanted tissue is also shorter than maternal and recipient cfDNA, indicating this property is not specific to ctDNA and can occur in different types of cfDNA [31,32].

A large amount of work in the cfDNA field has been focused in maternal-foetal medicine, and here it was observed that the amount of foetal DNA found in the mother’s blood correlates with the stage of development of the fetus – the further in development, the greater the foetal cfDNA content [33,34]. This has also been shown to be true of tumours where the size [35,36] and stage [37,38] correlates with the amount of ctDNA. Stage IV or advanced tumours have 100–1000 copies of ctDNA per 5 mL of plasma, compared with only 10 copies in early stage cancers [38]. Additional increases are also seen in patients with metastatic disease. Parkinson et al. attempted to quantify this in patients with relapsed high-grade serous ovarian carcinoma (HGSOC) and found an increase of 6 copies of ctDNA per mL plasma for additional each cm 3 of disease [39].

Not much is currently known about the function of ctDNA, however several studies have indicated possible roles in immunomodulation [40–42] or transformation of nearby cells through internalization of ctDNA [43]. The latter studies have yet to be confirmed in an in vivo setting, however NIH-3T3 cell lines merely coming into contact with KRAS-mutant plasma have been transformed in vitro [44]. There remains much more to learn about the function of ctDNA and cfDNA, which hopefully will be revealed through additional research.

Methodologies for cfDNA analysis

PCR-based techniques

The first ctDNA matching a patient's tumour mutation was identified using allele-specific PCR in 1994 [45]. Although still used in the field, digital PCR, which involves single-molecule reactions, is favoured as it provides absolute quantification of the number of transcripts in a sample [46,47]. This development has allowed the detection of rare mutations even at low abundance and has significantly increased the sensitivity of ctDNA analysis [48–50]. Droplet digital PCR (ddPCR) involves the distribution of DNA templates into thousands or millions of droplets each containing only one DNA fragment. There are many different commercially available ddPCR platforms that use various numbers of droplets and slightly different chemistries [51]. These assays mostly rely on fluorescent probes and can be multiplexed to detect the presence of a few loci or hotspots at a time. ddPCR has the potential to be applied in the clinic to monitor tumour dynamics and in the detection of minimal residual disease [52]. Despite the limitations in the number of mutations that can be tested for at a time, ddPCR is still a regularly used cfDNA technology as it provides quick turnaround time and high levels of sensitivity compared to sequencing strategies.

Next-generation sequencing-based techniques

Next-generation sequencing (NGS) techniques provide the opportunity to discover mutations without prior knowledge of the tumour mutation landscape. One type of sequencing strategy is amplicon based, where primers are designed to amplify hundreds of regions and sequenced to very high coverage to achieve a 1% sensitivity for de novo mutation calling or 0.1% sensitivity for genotyping [53,54]. Many of these panels are designed to be patient or cancer specific based on prior knowledge of the mutations within the tumour or using literature findings from tumour profiling. One key advantage of an amplicon-based strategy is the high efficiency of converting cfDNA molecules into sequencing reads, as a result of the high efficiency of amplification by PCR. Although due to challenges in retaining the relative ratio between genomic regions throughout the PCR amplification process, this method has less sensitivity for detecting copy number alterations.

Another NGS strategy is hybridisation capture-based approaches. Larger regions of the genome can be interrogated, however there is a trade-off between the breadth and depth of sequencing for the same cost. As a result, the sequencing coverage is generally reduced in larger panels leading to lower sensitivity. More sensitive patient specific assays can be designed containing only a few genes or loci of interest, however more commonly panels are developed to include multiple genes with important prognostic and diagnostic information for a specific cancer type. Cancer personalized profiling by deep sequencing (CAPP-Seq) is an example of this, and was first used to build a panel for non-small cell lung cancer (NSCLC) [55]. Larger panels encompassing hundreds of genes across many cancer types also exist and panels derived for tumour profiling can be adapted for cfDNA [56–58]. More recently, whole exome sequencing has been done on cfDNA samples, allowing identification of additional mutations and resistance mechanisms [59–63]. One of the major improvements in hybrid capture sequencing technology over the last few years has been the introduction of

uniquemolecular identifiers (UMIs). UMIs are added to sequencing adaptors early in the library preparation to tag each template DNA fragment with a unique molecular barcode [64]. Once barcoded, any PCR products and reads deriving from that template will retain the barcode allowing them to be identified and collapsed into a consensus sequence, minimizing artefacts arising from PCR and sequencing errors. Many cfDNA sequencing strategies have successfully been adapted to incorporate UMIs [65–67].

Apart from point mutations and indels, epigenetic changes and chromosomal aberrations cannot be easily detected using capture-based methods. As these could also be drivers of cancer, methods designed specifically to detect these changes have been developed or adapted for cfDNA. For instance, bisulphite or other methylation sequencing methods can be used on cfDNA [24,26,68].ctDNA studies involving methylation analysis have included methylation specific PCRs [69] , arrays [70] , and bisulphite sequencing [71–73]. For copy number, the two main methods that have been employed are ddPCR [74–78] and whole genome sequencing at very low coverage (~0.1X), known as shallow whole genome sequencing (sWGS) [79] or Plasma-Seq [80,81]. By comparing the coverage across the genome to normal samples, chromosomal alterations originating from ctDNA can be detected and quantified [82], including focal amplifications and gene rearrangements. Computational modelling approaches have been developed using sWGS data to estimate the proportion of ctDNA within the cfDNA sample [81,83], a concept similar to tumour purity in biopsy samples. However, in this case knowing the fraction of molecules derived from the tumour can be used to identify patients with high disease burden [81] and to screen samples for techniques that do not require very high sensitivity and can provide additional information, such as exome sequencing [60]. A summary of the features and applications of PCR-based and NGS approaches for cfDNA can be found in Table 1.

Method sensitivity

By comparing the number of mutant alleles to wildtype alleles for multiple loci the average mutant allele fraction (MAF) within the sample can be derived, a metric which correlates with tumour burden [84]. The MAF will vary between alleles are they are represented at different proportions due to tumour heterogeneity. Those at higher allele fractions are likely stem mutations, while lower allele fractions are likely private mutations [84,85], although factors such as copy number can also influence the allele fraction. Despite these differences in allelic frequency, the average MAF of a sample still correlates with disease volume. Parkinson et al. managed to quantify this for HGSOC and found the MAF increases by 0.08% for each additional cm 3 of disease [39].

The sensitivity of the technologies varies greatly, and a useful way of expressing that sensitivity is using the range of MAF they are able to detect. sWGS requires between 5–10% MAF to be detected [81], very high amounts for cfDNA, and cannot generally be used for detecting early or residual disease. However, this is dependent on cancer type: early HGSOC, which often presents with a high number of copy number variants (CNVs), has been assessed using sWGS with CNVs detected in 6/16 patients (37.5%) [86]. Other cancer types, which do not have large CNVs, may not be detected using this strategy.

Larger panels and exome sequencing have a similar limit of detection of 5% MAF [62], while smaller multiplexed panels can detect mutations down to 1% MAF [56,87,88]. This can be further improved by using cancer or patient specific panels, which can detect 0.01–0.5% MAFs. The CAPP-Seq assay for NSCLC, for instance, has a MAF limit of 0.02% and was able to find mutations in 100% of stage II or greater cancers, and in 50% of stage I [55].

PCR based assays have very high sensitivity; qPCR and ddPCR are able to detect 0.001–0.01% MAF [89], and in some samples only one copy in 5 mLs of plasma [90]. When compared with known mutation status based on tumour assays, KRAS ctDNA assays developed for clinically relevant mutations within colorectal cancer achieved 87.2% sensitivity and 99.2% specificity [91] and another similar assay developed against known mutations in early-stage breast cancer reached 93.3% sensitivity [92].

Using known mutations identified within a patient's tumour sample can also improve the sensitivity of NGS assays [18,93] and reduce the background error rate [53]. As evidence for the mutation already exists, it is easier to be confident calling mutations in the corresponding cfDNA. As discussed above, using UMIs when making sequencing libraries increases sensitivity and can allow detection of MAFs down to 0.1% [54,65,66]. Another method to improve ctDNA detection is through size selection. As previously discussed, ctDNA is shorter than normal cfDNA. Physical size selection of the shorter molecules has been shown to enrich the sample for tumour derived fragments and improve detection rates after sequencing [29,94,95]. These strategies are just beginning to be explored, but they have to potential to significantly increase the sensitivity of these types of assays and making them a possibility for use in the clinic.

Factors affecting mutant allele fraction

Although high levels of ctDNA is correlated with high disease burden, pre-analytical, analytical and physiological factors can affect the amount ctDNA fraction within a cfDNA sample. cfDNA undergoes quick turnover in the blood [13,19,20] , reducing overall yields. Additionally, release of DNA from blood cells increases the normal cfDNA content diluting ctDNA within the sample. Specialized blood collection tubes, such as Streck and PAXgene cfDNA tubes, have been developed to protect cfDNA from degradation extending the time samples can be stored and limiting DNA release [96–98]. These are an easy and inexpensive way to ensure cfDNA remains intact and ctDNA within the sample is undiluted. Location and vascularization of the tumour can also affect ctDNA release into the bloodstream. This is most commonly observed in patients with primary brain tumours, where low amounts of ctDNA within the blood is thought to be due to the blood brain barrier [38,99]. To overcome this limitation, cfDNA samples can be obtained from CSF, which yields better results than plasma [100]. This strategy can also be used in patients with brain metastasis and central nervous system restricted disease [101]. This strategy also applies to other tumour types, cfDNA can be extracted from other liquid sample sources in close proximity to the tumour, for instance through collecting urine [15,102] or saliva [103] to increase the tumour-derived fraction.

Clinical need

cfDNA analysis will not replace tumour biopsies, however they can provide additional insight into and important clinically relevant information about the mutational landscape of tumours and metastases. In cases where it is not possible to biopsy or repeated biopsies are required cfDNA analyses are of enormous benefit, as they can provide information not otherwise obtainable. Furthermore, there are additional benefits to using cfDNA when biopsies have been taken, for instance for tumour heterogeneity, metastasis, and longitudinal studies.

Tumour heterogeneity and metastatic sites

Tumours are often heterogeneous and can have regional differences in mutation profiles [104–107] leading to actionable or prognostic mutations being missed during biopsy. cfDNA can capture these mutations to provide clinically relevant information missed due to heterogeneity [56,84,85,100,108,109], and identify mutations missed during sequencing of tumour biopsies [110,111]. This is also true of multiple tumours [112] or metastatic sites [113] ; variants missed due to spatial separation from the biopsy site can be detected using cfDNA [59,87,114].

Identifying tissue of cancer origin

Studies of DNA methylation can be used to identify the tissue of origin, a process that is especially important in cancers of unknown primary [115], and cfDNA methylation and nucleosome positioning studies can be used the same way [24–26,116]. For instance, a pregnant woman undergoing non-invasive prenatal testing was found to have copy number changes not associated with the fetus , methylation deconvolution indicated an increased contribution from B-lymphocytes and she was subsequently given a diagnosis of follicular lymphoma [117]. Using cfDNA can provide insight into the tumour tissue type, without requiring a biopsy.

Measuring disease burden

The presence of ctDNA itself is indicative of disease and the amount of ctDNA can also be an indicator of the amount of disease. As previously discussed, the amount of ctDNA is correlated with tumour stage, and many groups have observed that higher levels of ctDNA has been associated with worse survival outcomes in patients [69,118–122]. Thus, the amount of ctDNA can be used as a measure disease burden, along with imaging studies. In a colorectal cancer study, patients with detectable ctDNA after treatment had reduced (48%) 2-year recurrence free survival compared to those without (100%) [69]. Similarly, in prostate cancer patients who had AR copy number gains detected by cfDNA analysis prior to abiraterone treatment had worse outcomes and exhibited primary resistance [77]. Lower ctDNA levels correlate with treatment response [18,53,55,114,120] and this response can be detected earlier than with clinical detection methods [114,123,124]. This provides additional information about how a patient will respond to treatment. Overall, ctDNA has been shown to be a better predictor of prognosis than other tumour markers [39,114] and that ctDNA concentration increase correlates with poorer clinical and radiological outcomes [39,114,125,126].

Patient stratification

Total levels of ctDNA provide insight into disease burden and prognosis, but more specific molecular studies can provide the underlying genetic profile. Using molecular profiling tests prior to treatment provides the possibility of stratifying patients based on prognosis for the administration of adjuvant therapy [77,124] or for selection of specific targeted therapies.

Tracking specific mutations

Specific mutations can also be tracked during treatment to signal recurrence or relapse of the tumour before clinical evidence of disease, as is possible with total ctDNA burden [120]. On average, ctDNA can be detected between 7.9 and 11.0 months prior to clinical relapse in primary cancers [127–129], with similar results being found for metastases [114]. These types of assays require very sensitive approaches such as ddPCR, rely on previous knowledge of the tumour mutation profile, and are developed in a patient specific manner. This poses a challenge for clinical development as individual assays need to be created and optimized for each patient, however they have been shown in research to be very powerful methods for detecting even minimal residual disease. In a large study of early-stage colorectal cancer, ctDNA levels measured at follow up after tumour resection was predictive of recurrence-free survival after 3 years. In the ctDNA positive group 0% were recurrence-free, while in the ctDNA negative group 90% were recurrence-free, this number was improved to 100% after serial samples were taken into account [130].ctDNA positivity is indicative of minimal residual disease, the level of which can be tracked and quantified through these types of assays. A similar study was completed on patients with breast cancer, where personalized assays were able to predict recurrence and measure minimal residual disease [127]. This can also be done using patient specific rearrangements such as gene fusions [128,131,132], as rearrangements have low background noise compared to somatic mutations.

Detecting tumour evolution and acquisition of resistance

Tracking recurrence of individual mutations or rearrangements present in the original tumour provides useful information for recurrence of tumour cells containing that mutation, but does not provide additional information about recurrence with new mutations or emergence of other clones. By measuring several mutations, the changes in their ratios can provide some insight into the tumour’s evolution and continued heterogeneity during treatment [84,133–135]. This can also be extended to identifying the appearance of resistance mechanisms. Serial studies of colorectal cancer found positive selection of KRAS mutations during anti-EGFR therapy and decline in their representation after withdrawal [108,136]. Similar results have been recorded in NSCLC patients treated with EGFR inhibitors, where resistance mutations were identified in ctDNA prior to clinical progression [50,137,138]. Another study completed this year identified resistance mutations not previously observed in the tumour as driving resistance to treatment [139], indicating convergent evolution was taking place and could be identified using ctDNA. Additional studies are currently on-going involving exome sequencing to discover novel resistance mechanisms in ctDNA developed during treatment [59,84], however as these mutations need

to be present at > 5% MAF to be detected, this is not a technique that can be used for low frequency variants or for minimal residual disease.

Clinical development

Non-invasive prenatal testing

There's a huge potential for cfDNA in the clinic and already many cfDNA assays have made an impact. The initial assays were developed for identifying foetal cfDNA sequences in maternal blood plasma. This included finding foetal Y chromosome cfDNA in maternal blood, indicating a male fetus, and other tests for sex determination [33]. Non-invasive sex determination is particularly important for carriers of X-linked conditions, and cfDNA testing has resulted in a decrease in the number of invasive tests, such as amniocentesis, to confirm foetal sex [140]. Detection of chromosomes can also be done to discover aneuploidy, and has successfully been used to detect Down Syndrome (trisomy 21) [74], a test that was made available in the clinic in 2011 [141–143] and is now in widespread clinical use [144,145]. These techniques were refined to look at specific gene sequences and cfDNA tests have been developed monogenic disorders as well [146].cfDNA studies in maternal-foetal medicine have paved the way for the development of more highly specific clinical cancer assays.

PCR for guiding treatment

The most famous example of a clinically validated cfDNA test is the allele-specific PCR for the detection of EGFR mutations in NSCLC [147]. Plasma has been shown to be effective and comparable to tissue biopsies in NSCLC [148–150] and as the tumours can be difficult to biopsy [151], clinical approval has been driven faster than for other types of cancer. Two forms of the test exist and are approved for selection of gefitinib [152], erlotinib [153] or osimertinib [153,154]: Therascreen EGFR was approved by the European Medicines Agency in 2015 [155] and Cobas EGFR was approved by the US Food and Drug Administration (FDA) in 2016 [153].PCR-based tests are highly sensitive and specific, are easier to gain approval for than NGS assays, and additional ctDNA assays for other genes and cancer types are likely to follow soon.

Sequencing panels for guiding treatment

Although the majority of clinical development has been in PCR-based assays, some clinical sequencing panels have been developed. A 34 gene panel developed by Inviata for NSCLC found mutations in 79% of 174 patients, following this 28 patients (17%) went on to receive personalized treatment [156]. Another broader 54-gene panel detected ctDNA in 58% of patients with multiple types of cancer, with 68% of those having an actionable mutation by an FDA-approved drug [57]. Similar proof of concept studies have been done showing the feasibility of matching metastatic patients to targeted therapies for clinical trials, however they have not been approved as of yet [157]. Numerous companies have begun to capitalize on the prospect of ctDNA screening, including Guardant Health, who have developed a 73-gene ctDNA assay called Guardant 360 [67,158]. Assays such as these have begun to be used to guide enrolment in clinical trials [159], in particular for cases where a positive finding results in enrolment.

Screening for early detection of disease

cfDNA analysis has the potential to be used as a screening tool prior to clinical onset of disease [103,160,161]. Screening of viruses associated with cancers, such as Epstein–Barr Virus (EBV) – a virus associated with nasopharyngeal carcinoma, has been shown to be an effective way to use cfDNA to screen for early stage and asymptomatic cancers in the clinic, as viral cfDNA levels are much higher than ctDNA. One study involved screening of > 20,000 asymptomatic people for EBV DNA in their plasma and lead to the diagnosis of nasopharyngeal carcinoma in 34 individuals [162]. Identification of these patients led to early diagnosis and improved 3-year progression-free survival [162]. Screening for ctDNA is also being trialled, recently a screening panel of 58 genes has been developed for early detection of cancer [161], although it has only been used in patients with known stage I or II disease to date, this study demonstrates the potential of screening ctDNA. Techniques such as this have the potential to improve patient outcomes and continued development will soon improve non-invasive cancer screening.

Future developments

Sequencing developments

Promising improvements are being made in sequencing technology and bioinformatics methods that will benefit and improve cfDNA analysis. Longer cfDNA fragments are not easily detected during current sequencing methods [163], however long read sequencers, such as Nanopore, have improved ability to identify structural variants [164]. Additionally, using alternative library preparation methods, such as single-stranded library preparation, has been shown to increase the diversity of sequenced fragments to include the shorter molecules often missed during standard double stranded preparations [24,165,166]. These methods could also allow further study of cfDNA fragmentation, which could provide complementary information to MAF for determining the amount of ctDNA in a sample [88,167] or to understand the fragmentation and clearance from blood through urine [20,168]. Additional studies into the longer and shorter fragments could provide additional insight about the biology of cfDNA and ctDNA.

Advances in biological knowledge

Although it is known that ctDNA is shorter than cfDNA, the exact mechanism of how this occurs has yet to be elucidated. Studies into these size differences, as well as how cfDNA is protected in body fluids would provide important information about these mechanisms as well as tumour specific processes resulting in the release of ctDNA. There are numerous factors influencing the amount of cfDNA in circulation and the proportion of ctDNA other than the size of the tumour. The amount of exercise done prior to blood draw can increase the amount of lymphocyte derived cfDNA greatly [169,170], which dilutes the ctDNA within a sample making detection of mutations more difficult. However, why and how this happens remains unknown. Additional factors, such as blood supply to the tumour presumably could also have an effect on the amount of ctDNA in the blood, and again these factors have not been studied in detail.

Research by Snyder et al. indicates an association between cfDNA and nucleosomes, however functional studies have not yet been done to confirm how long this association lasts and if recovered cfDNA is still bound [24]. Several groups have also suggested studies should be done into the other proteins associated with cfDNA [171,172], however little research has been done into this area as of yet outside of studies of exosomes.

Exosomes and cell-free RNA

Exosomes are membrane bound vesicles containing proteins, DNA, and RNA that is packaged and excreted from cells for transport or signalling between cells [14,173]. After being excreted, exosomes can enter bodily fluids, such as blood, and can subsequently be isolated and purified. Exosomes can be a rich source of information often lost from other cell-free samples as the membrane protects the contents within from degradation. They may contribute to cancer progression and treatment failure, and can contain ctDNA [173–175]. Numerous studies of the content of exosomes have been published [176,177], including combined cfDNA and cell-free RNA (cfRNA or exRNA) studies [178]. Exosomes are a promising way to study cfRNA [178,179]. Sequencing of the exosomal RNA from plasma in normal healthy individuals [180,181] and cancer patients [182,183], has identified that the majority of RNA within exosomes is microRNA (miRNA), with some fragments of coding sequence (mRNA) present as well.

RNA can also be detected at a lower level in a cell-free state in plasma without the time consuming and difficult procedures of isolating exosomes. cfRNA extracted this way also mainly contains miRNAs [184], however, important diagnostic and prognostic mRNA fragments can also be identified [185–187].Sequencing of cfRNA has been done by several groups [188–190], although the most promising avenue currently being explored for cancer is in the identification of tumour specific RNA transcripts and fusion genes, which are especially difficult to detect through DNA methods. That said, by further sequencing and characterizing of the exosomal and cfRNA profiles, it is possible that they could provide more information about cancer both for diagnostics and for fundamental studies of cancer biology and progression.

Clinical progress

Progress in the development in clinical tests and validation has been rapid, and is likely to continue in that fashion as they are approved in additional cancer types and as more drugs are developed requiring molecular stratification. The only approved tests currently use plasma as a starting source for cfDNA, however there is potential for this to expand to other sources of cfDNA and to cfRNA.

Most studies are currently focused on identification of mutations and cancer evolution; although many other potential areas of research and clinical use exist that have yet to be explored in depth, such as in understanding response to immunotherapy [120,125,191]. Further studies into these more complex areas will be able to provide additional information, not only about cancer, but also about cfDNA itself, and will help us to fully understand the potential and limitations of cfDNA analysis.

Conclusion

cfDNA analysis is a non-invasive method for obtaining important and clinically relevant molecular information about cancer that is rapidly gaining momentum in the research and clinical world. Technological developments in molecular biology and NGS approaches continue to advance the sensitivity of cfDNA analysis techniques, increasing the potential for its use not just in prognostic and diagnostic settings where disease burden is high, but also for minimal residual disease, emergence of resistance, and early screening. Clinically validated cfDNA assays are beginning to make an appearance in oncology, and will likely become widespread for monitoring response to treatment and molecular stratification. As research into additional sources of cfDNA and cell-free analytes continues, a wider variety of options for detection will become available and an increased understanding of the biology of cfDNA and ctDNA can be gained.

Funding statement

We acknowledge the Department of Pathology and the MarieJosé and Henry R. Kravis Center for Molecular Oncology of the Memorial Sloan Kettering Cancer Center, and Memorial Sloan Kettering Cancer Center Support Grant (NIH/NCI, Grant No P30CA008748) for their support of the authors.

Role of study sponsors

The sponsors have no involvement in the preparation of the manuscript and decision to submit.

References

[1]. Jahr S, 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(4):1659–65. [PubMed: 11245480]

[2]. Stroun M, et al. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta 2001;313(1–2):139–42. [PubMed: 11694251]

[3]. Mandel P, Metais P.Les acides nucleiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 1948;142(3–4):241–3. [PubMed: 18875018]

[4]. Zhang J, et al. Presence of donorand recipient-derived DNA in cell-free urine samples of renal transplantation recipients: urinary DNA chimerism. Clin Chem 1999;45(10):1741–6. [PubMed: 10508119]

[5]. Sidransky D, et al. Identification of p53 gene mutations in bladder cancers and urine samples. Science 1991;252(5006):706–9. [PubMed: 2024123]

[6]. Rhodes CH, Honsinger C, Sorenson GD.Detection of tumor-derived DNA in cerebrospinal fluid. J Neuropathol Exp Neurol 1994;53(4):364–8. [PubMed: 7912724]

[7]. Sriram KB,et al. Pleural fluid cell-free DNA integrity index to identify cytologically negative malignant pleural effusions including mesotheliomas. BMC Cancer 2012;12(1):428. [PubMed: 23009708]

[8]. Wang Y, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med 2015;7(293):293ra104.

[9]. Mithani SK, et al. Mitochondrial resequencing arrays detect tumor-specific mutations in salivary rinses of patients with head and neck cancer. Clin Cancer Res 2007;13(24):7335. [PubMed: 18094415]

[10]. Nadano D, Yasuda T, Kishi K.Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. Clin Chem 1993;39(3):448–52. [PubMed: 8448855]

[11]. Tamkovich SN, et al. Circulating DNA and DNase activity in human blood. Ann N Y Acad Sci 2006;1075:191–6. [PubMed: 17108211]

[12]. Yao W, et al. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: a qualitative study. Gene 2016;590(1):142–8. [PubMed: 27317895]

[13]. Chused TM, Steinberg AD, Talal N.The clearance and localization of nucleic acids by New Zealand and normal mice. Clin Exp Immunol 1972;12(4):465–76. [PubMed: 4650369]

[14]. Thierry AR, et al. Origins, structures, and functions of circulating DNA in oncology. Cancer Metastasis Rev 2016;35(3):347–76. [PubMed: 27392603]

[15]. Botezatu I, et al. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem 2000;46(8):1078–84. [PubMed: 10926886]

[16]. Bryzgunova OE, Laktionov PP.Extracellular nucleic acids in urine: sources, structure, diagnostic potential. Acta Nat 2015;7(3):48–54.

[17]. Chan KCA, et al. Quantitative analysis of the transrenal excretion of circulating EBV DNA in nasopharyngeal carcinoma patients. Clin Cancer Res 2008;14(15):4809. [PubMed: 18676752]

[18]. Diehl F, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14(9):985–90. [PubMed: 18670422]

[19]. Lo YM, et al. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64(1): 218–24. [PubMed: 9915961]

[20]. Yu SC, et al. High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. Clin Chem 2013;59(8):1228–37. [PubMed: 23603797]

[21]. Jiang P, Lo YM.The long and short of circulating cell-free DNA and the ins and outs of molecular diagnostics. Trends Genet 2016;32(6):360–71. [PubMed: 27129983]

[22]. Giacona MB, et al. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas 1998;17(1):89–97. [PubMed: 9667526]

[23]. Lo YM, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med 2010;2(61):61ra91.

[24]. Snyder MW, et al. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. Cell 2016;164(1–2):57–68. [PubMed: 26771485]

[25]. Sun K, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. Proc Natl Acad Sci 2015;112(40):E5503–12. [PubMed: 26392541]

[26]. Lehmann-Werman R, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. Proc Natl Acad Sci U S A 2016;113(13):E1826–34. [PubMed: 26976580]

[27]. Thierry AR, et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res 2010;38(18):6159–75. [PubMed: 20494973]

[28]. Mouliere F, et al. High fragmentation characterizes tumour-derived circulating DNA. PLOS ONE 2011;6(9):e23418. [PubMed: 21909401]

[29]. Underhill HR, et al. Fragment length of circulating tumor DNA. PLOS Genet 2016;12(7):e1006162. [PubMed: 27428049]

[30]. Jiang P, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci 2015;112(11):E1317–25. [PubMed: 25646427]

[31]. Yu SCY, et al. Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. Proc Natl Acad Sci 2014;111(23):8583–8. [PubMed: 24843150]

[32]. Zheng YW, et al. Nonhematopoietically derived DNA is shorter than hematopoietically derived DNA in plasma: a transplantation model. Clin Chem 2012;58(3):549–58. [PubMed: 22052939]

[33]. Lo YM, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350(9076):485–7. [PubMed: 9274585]

[34]. Lo YM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62(4):768–75. [PubMed: 9529358]

[35]. Gorges TM, et al. Cancer therapy monitoring in xenografts by quantitative analysis of circulating tumor DNA. Biomarkers 2012;17(6):498–506. [PubMed: 22616911]

[36]. Kamat AA, et al. Circulating cell-free DNA: a novel biomarker for response to therapy in ovarian carcinoma. Cancer Biol Ther 2006;5(10):1369–74. [PubMed: 16969071]

[37]. Nawroz H, et al. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996;2(9):1035–7. [PubMed: 8782464]

[38]. Bettegowda C, et al. Detection of circulating tumor DNA in earlyand late-stage human malignancies. Sci Transl Med 2014;6(224):224ra24.

[39]. Parkinson CA, et al. Exploratory analysis of TP53 mutations in circulating tumour DNA as biomarkers of treatment response for patients with relapsed high-grade serous ovarian carcinoma: a retrospective study. PLoS Med 2016;13(12):e1002198. [PubMed: 27997533]

[40]. Kostyuk SV, et al. Fragments of cell-free DNA increase transcription in human mesenchymal stem cells, activate TLR-dependent signal pathway, and suppress apoptosis. Biochem Suppl Ser B: Biomed Chem 2012;6(1):68–74.

[41]. Nishimoto S, et al. Obesity-induced DNA released from adipocytes stimulates chronic adipose tissue inflammation and insulin resistance. Sci Adv 2016;2:3.

[42]. Dvorakova M, et al. DNA released by leukemic cells contributes to the disruption of the bone marrow microenvironment. Oncogene 2013;32(44):5201–9. [PubMed: 23222712]

[43]. Gartler SM.Cellular uptake of deoxyribonucleic acid by human tissue culture cells. Nature 1959;184(4697):1505–6. [PubMed: 13826696]

[44]. Garcia-Olmo DC, et al. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. Cancer Res 2010;70(2):560–7. [PubMed: 20068178]

[45]. Sorenson GD, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomark Prev 1994;3(1):67–71.

[46]. Vogelstein B ,Kinzler KW.Digital PCR. Proc Natl Acad Sci 1999;96(16):9236–41. [PubMed: 10430926]

[47]. Dressman D, et al. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc Natl Acad Sci 2003;100(15):8817–22. [PubMed: 12857956]

[48]. Schiavon G, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med 2015;7(313):313ra182.

[49]. Taly V, et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem 2013;59(12):1722. [PubMed: 23938455]

[50]. Sacher AG, et al. prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer. JAMA Oncol 2016;2(8):1014–22. [PubMed: 27055085]

[51]. Baker MDigital PCR hits its stride. Nat Meth 2012;9(6):541–4.

[52]. Yung TK, et al. 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(6):2076–84. [PubMed: 19276259]

[53]. Forshew T, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4(136):136ra68.

[54]. Gale D, et al. Analytical performance and validation of an enhanced TAm-Seq circulating tumor DNA sequencing assay. Inviata 2016.

[55]. Newman AM, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20(5):548–54. [PubMed: 24705333]

[56]. De Mattos-Arruda L, 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(9):1729–35. [PubMed: 25009010]

[57]. Schwaederle M, et al. Detection rate of actionable mutations in diverse cancers using a biopsy-free (blood) circulating tumor cell DNA assay. Oncotarget 2016;7(9):9707–17. [PubMed: 26848768]

[58]. Pal SK, et al. Evolution of circulating tumor DNA profile from first-line to subsequent therapy in metastatic renal cell carcinoma. Eur Urol 2017;2(4):557–64.

[59]. Butler TM, et al., Exome sequencing of cell-free DNA from metastatic cancer patients identifies clinically actionable mutations distinct from primary disease. PLoS One, 2015 10(8): p. e0136407. [PubMed: 26317216]

[60]. Adalsteinsson VA, et al. Scalable whole-exome sequencing of cell-free DNA reveals high concordance with metastatic tumors. Nat Commun 2017;8(1):1324. [PubMed: 29109393]

[61]. Chicard M, et al. Whole exome sequencing of cell-free DNA reveals temporo-spatial heterogeneity and identifies treatment-resistant clones in neuroblastoma. Clin Cancer Res 2017;24(4):939–49. [PubMed: 29191970]

[62]. Murtaza M, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497(7447):108–12. [PubMed: 23563269]

[63]. Dietz S, et al. Low input whole-exome sequencing to determine the representation of the tumor exome in circulating DNA of non-small cell lung cancer patients. PLoS One 2016;11(8):e0161012. [PubMed: 27529345]

[64]. Kivioja T, et al. Counting absolute numbers of molecules using unique molecular identifiers. Nat Methods 2012;9(1):72–4.

[65]. Kinde I, et al. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A 2011;108:9530–5. [PubMed: 21586637]

[66]. Newman AM, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol 2016;34(5):547–55. [PubMed: 27018799]

[67]. Lanman RB, et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. PLOS ONE 2015;10(10):e0140712. [PubMed: 26474073]

[68]. Ørntoft M-BW, et al. Comparative analysis of 12 different kits for bisulfite conversion of circulating cell-free DNA. Epigenetics 2017;12(8):626–36. [PubMed: 28557629]

[69]. Lecomte T, et al. Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. Int J Cancer 2002;100(5):542–8. [PubMed: 12124803]

[70]. Melnikov AA, et al. Methylation profile of circulating plasma DNA in patients with pancreatic cancer. J Surg Oncol 2009;99(2):119–22. [PubMed: 19065635]

[71]. Kadam SK, Farmen M, Brandt JT.Quantitative measurement of cell-free plasma DNA and applications for detecting tumor genetic variation and promoter methylation in a clinical setting. J Mol Diagn 2012;14(4):346–56. [PubMed: 22579630]

[72]. Chan KC, et al. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proc Natl Acad Sci U S A 2013;110(47):18761–8. [PubMed: 24191000]

[73]. Zhao Y, et al. Genome-wide methylation profiling of the different stages of hepatitis B virus-related hepatocellular carcinoma development in plasma cell-free DNA reveals potential biomarkers for early detection and high-risk monitoring of hepatocellular carcinoma. Clin Epigenet 2014;6(1):30.

[74]. Lo YM, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A 2007;104(32):13116–21. [PubMed: 17664418]

[75]. Miotke L, et al. High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR. Anal Chem 2014;86(5):2618–24. [PubMed: 24483992]

[76]. Gevensleben H, et al. Noninvasive detection of HER2 amplification with plasma DNA digital PCR. Clin Cancer Res 2013;19(12):3276–84. [PubMed: 23637122]

[77]. Romanel A, et al. Plasma AR and abiraterone-resistant prostate cancer. Sci Transl Med 2015;7(312):312re10.

[78]. Garcia-Murillas I, Lambros M, Turner NC.Determination of HER2 amplification status on tumour DNA by digital PCR. PLOS ONE 2013;8(12):e83409. [PubMed: 24386193]

[79]. Chin SF, et al. Shallow whole genome sequencing for robust copy number profiling of formalin-fixed paraffin-embedded breast cancers. bioRxiv 2017.

[80]. Heitzer E, et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013;5(4):30. [PubMed: 23561577]

[81]. Belic J, et al. Rapid identification of plasma DNA samples with increased ctDNA levels by a modified FAST-SeqS approach. Clin Chem 2015;61(6):838–49. [PubMed: 25896989]

[82]. Leary RJ, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4(162):162ra154.

[83]. Chan KCA, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem 2013;59(1):211. [PubMed: 23065472]

[84]. Murtaza M, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. Nat Commun 2015;6:8760. [PubMed: 26530965]

[85]. Jamal-Hanjani M, et al. Detection of ubiquitous and heterogeneous mutations in cell-free DNA from patients with early-stage non-small-cell lung cancer. Ann Oncol 2016;27(5):862–7. [PubMed: 26823523]

[86]. Cohen PA, et al. Abnormal plasma DNA profiles in early ovarian cancer using a non-invasive prenatal testing platform: implications for cancer screening. BMC Med 2016;14(1):126. [PubMed: 27558279]

[87]. Lebofsky R, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol 2015;9(4):783–90. [PubMed: 25579085]

[88]. Frenel JS, et al. Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecular ly targeted drug administration. Clin Cancer Res 2015;21(20):4586–96. [PubMed: 26085511]

[89]. Zonta E, et al. Multiplex detection of rare mutations by picoliter droplet based digital PCR: sensitivity and specificity considerations. PLOS ONE 2016;11(7):e0159094. [PubMed: 27416070]

[90]. Kidess E, et al. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget 2015;6(4):2549–61. [PubMed: 25575824]

[91]. Thierry AR, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med 2014;20(4):430–5. [PubMed: 24658074]

[92]. Beaver JA, et al. Detection of cancer DNA in plasma of patients with early-stage breast cancer. Clin Cancer Res 2014;20(10):2643–50. [PubMed: 24504125]

[93]. Diehl F, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A 2005;102(45):16368–73. [PubMed: 16258065]

[94]. Efficient generation of cfDNA libraries that are highly enriched in short fragments In: Proceedings of the advances in genome biology and technology (AGBT), Scottsdale, Arizona Sage Science and Rubicon Genomics; 2016.

[95]. Mouliere F, et al. Selecting short DNA fragments in plasma improves detection of circulating tumour DNA. bioRxiv 2017.

[96]. Medina Diaz I, et al. Performance of streck cfDNA blood collection tubes for liquid biopsy testing. PLOS ONE 2016;11(11):e0166354. [PubMed: 27832189]

[97]. Warton K, et al. Evaluation of streck BCT and PAXgene stabilised blood collection tubes for cell-free circulating DNA studies in plasma. Mol Diagn Ther 2017;21(5):563–70. [PubMed: 28631163]

[98]. Parpart-Li S, et al. The effect of preservative and temperature on the analysis of circulating tumor DNA. Clin Cancer Res 2017;23(10):2471–7. [PubMed: 27827317]

[99]. Pan W, et al. Brain tumor mutations detected in cerebral spinal fluid. Clin Chem 2015;61(3):514–22. [PubMed: 25605683]

[100]. De Mattos-Arruda L, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. Nat Commun 2015;6:8839. [PubMed: 26554728]

[101]. Pentsova EI, et al. Evaluating cancer of the central nervous system through next-generation sequencing of cerebrospinal fluid. J Clin Oncol 2016;34(20):2404–15. [PubMed: 27161972]

[102]. Birkenkamp-Demtroder K, et al. Genomic alterations in liquid biopsies from patients with bladder cancer. Eur Urol 2016;70(1):75–82. [PubMed: 26803478]

[103]. Mao L, et al. Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. Cancer Res 1994;54(7):1634–7. [PubMed: 8137272]

[104]. Gerlinger M, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. New Engl J Med 2012;366(10):883–92. [PubMed: 22397650]

[105]. Yates LR, et al. Subclonal diversification of primary breast cancer revealed by multiregion sequencing. Nat Med 2015;21(7):751–9. [PubMed: 26099045]

[106]. de Bruin EC, et al. Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. Science 2014;346(6206):251–6. [PubMed: 25301630]

[107]. Zhang J, et al. Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. Science 2014;346(6206):256–9. [PubMed: 25301631]

[108]. Siravegna G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med 2015;21(7):795–801. [PubMed: 26030179]

[109]. Oxnard GR, et al. Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer. J Clin Oncol 2016;34(28):3375–82. [PubMed: 27354477]

[110]. De Mattos-Arruda L, et al. Establishing the origin of metastatic deposits in the setting of multiple primary malignancies: the role of massively parallel sequencing. Mol Oncol 2014;8(1):150–8. [PubMed: 24220311]

[111]. Kuo YB, et al. Comparison of KRAS mutation analysis of primary tumors and matched circulating cell-free DNA in plasmas of patients with colorectal cancer. Clin Chim Acta 2014;433:284–9. [PubMed: 24685572]

[112]. Criscitiello C, et al. Biopsy confirmation of metastatic sites in breast cancer patients: clinical impact and future perspectives. Breast Cancer Res 2014;16(2):205. [PubMed: 25032257]

[113]. Turajlic S, Swanton C.Metastasis as an evolutionary process. Science 2016;352(6282):169–75. [PubMed: 27124450]

[114]. Dawson SJ, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368(13):1199–209. [PubMed: 23484797]

[115]. Moran S, et al. Epigenetic profiling to classify cancer of unknown primary: a multicentre, retrospective analysis. Lancet Oncol 2016;17(10):1386–95. [PubMed: 27575023]

[116]. Kang S, et al. CancerLocator: non-invasive cancer diagnosis and tissue-of-origin prediction using methylation profiles of cell-free DNA. Genome Biol 2017;18:53. [PubMed: 28335812]

[117]. Bianchi DW, et al. Noninvasive prenatal testing and incidental detection of occult maternal malignancies. Jama 2015;314(2):162–9. [PubMed: 26168314]

[118]. Gautschi O, et al. Origin and prognostic value of circulating KRAS mutations in lung cancer patients. Cancer Lett 2007;254(2):265–73. [PubMed: 17449174]

[119]. Wang S, et al. Potential clinical significance of a plasma-based KRAS mutation analysis in patients with advanced non-small cell lung cancer. Clin Cancer Res 2010;16(4):1324–30. [PubMed: 20145159]

[120]. Gray ES, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget 2015;6(39):42008–18. [PubMed: 26524482]

[121]. Santiago-Walker A, et al. Correlation of BRAF mutation status in circulating-free DNA and tumor and association with clinical outcome across four BRAFi and MEKi clinical trials. Clin Cancer Res 2016;22(3):567–74. [PubMed: 26446943]

[122]. Zhuang R, et al. The prognostic value of KRAS mutation by cell-free DNA in cancer patients: a systematic review and metaanalysis. PLOS ONE 2017;12(8):e0182562. [PubMed: 28796802]

[123]. Marchetti A, et al. Early prediction of response to tyrosine kinase inhibitors by quantification of EGFR mutations in plasma of NSCLC patients. J Thorac Oncol 2015;10(10):1437–43. [PubMed: 26295376]

[124]. Scherer F, et al. Distinct biological subtypes and patterns of genome evolution in lymphoma revealed by circulating tumor DNA. Sci Transl Med 2016;8(364):364ra155.

[125]. Lipson EJ, et al. Circulating tumor DNA analysis as a real-time method for monitoring tumor burden in melanoma patients undergoing treatment with immune checkpoint blockade. J Immunother Cancer 2014;2:42. [PubMed: 25516806]

[126]. Oshiro C, et al. PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Treat 2015;150(2):299–307. [PubMed: 25736040]

[127]. Garcia-Murillas I, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015;7(302):302ra133.

[128]. Olsson E, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. EMBO Mol Med 2015;7(8):1034–47. [PubMed: 25987569]

[129]. Reinert T, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut 2016;65(4):625–34. [PubMed: 25654990]

[130]. Tie J, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016;8(346):346ra92.

[131]. Leary RJ, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci transl med 2010;2(20) pp. 20ra14–20ra14.

[132]. McBride DJ, et al. Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. Gen Chromosom Cancer 2010;49(11):1062–9.

[133]. Diaz LA, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486(7404):537–40. [PubMed: 22722843]

[134]. Misale S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 2012;486(7404):532–6. [PubMed: 22722830]

[135]. Chabon JJ, et al. Circulating tumour DNA profiling reveals heterogeneity of EGFR inhibitor resistance mechanisms in lung cancer patients. Nat Commun 2016;7:11815. [PubMed: 27283993]

[136]. Morelli MP, et al. Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. Ann Oncol 2015;26(4):731–6. [PubMed: 25628445]

[137]. Oxnard GR, 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(6):1698–705. [PubMed: 24429876]

[138]. Sorensen BS, et al. Monitoring of epidermal growth factor receptor tyrosine kinase inhibitor-sensitizing and resistance mutations in the plasma DNA of patients with advanced non-small cell lung cancer during treatment with erlotinib. Cancer 2014;120(24):3896–901. [PubMed: 25103305]

[139]. Thierry AR, et al. Circulating DNA demonstrates convergent evolution and common resistance mechanisms during treatment of colorectal cancer. Clin Cancer Res 2017;23(16):4578–91. [PubMed: 28400427]

[140]. Hyett JA, et al. Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. Prenat Diagn 2005;25(12):1111–16. [PubMed: 16231296]

[141]. Chiu RW, et al. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. Bmj 2011;342:c7401. [PubMed: 21224326]

[142]. Ehrich M, et al. Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting. Am J Obstet Gynecol 2011;204(3):205.e1–205.e11. [PubMed: 21310373]

[143]. Palomaki GE, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med 2011;13(11):913–20. [PubMed: 22005709]

[144]. Hill M, et al. Evaluation of non-invasive prenatal testing (NIPT) for aneuploidy in an NHS setting: a reliable accurate prenatal non-invasive diagnosis (RAPID) protocol. BMC Pregnancy Childbirth 2014;14:229. [PubMed: 25027965]

[145]. Allyse M, et al. Non-invasive prenatal testing: a review of international implementation and challenges. Int J Womens Health 2015;7:113–26. [PubMed: 25653560]

[146]. Saito H, et al. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. Lancet 2000;356(9236):1170. [PubMed: 11030304]

[147]. Normanno N, et al. Guide to detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with advanced non-small-cell lung cancer. Oncotarget 2017;8(7):12501–16. [PubMed: 27980215]

[148]. Douillard JY, et al. Gefitinib treatment in EGFR mutated caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. J Thorac Oncol 2014;9(9):1345–53. [PubMed: 25122430]

[149]. Han B, et al. Determining the prevalence of EGFR mutations in Asian and Russian patients (pts) with advanced non-small-cell lung cancer (NSCLC) of adenocarcinoma (ADC) and non-ADC histology: IGNITE study. Ann Oncol 2015;26:i29–30 suppl_1.

[150]. Reck M, et al. ctDNA determination of EGFR mutation status in European and Japanese patients with advanced NSCLC: the ASSESS study. J Thorac Oncol 2016;11(10):1682–9. [PubMed: 27468938]

[151]. Vanderlaan PA, et al. Success and failure rates of tumor genotyping techniques in routine pathological samples with non-small-cell lung cancer. Lung Cancer 2014;84(1):39–44. [PubMed: 24513263]

[152]. European Medicines Agency. Iressa: public assessment report — product information.European Medicines Agency, 2017 EMA 2016 21 10, Available from: http://www.ema.europa.eu/docsen_GB/document_library/EPAR__Product_Information/human/001016/WC500036358.pdf.

[153]. US Food and Drug Administration. Premarket approval P150044 — Cobas EGFR MUTATION TEST V2.US Food and Drug Administration, 2016 21 October 2017 Available from: http://www.accessdata.fda.gov/cdrh_docs/pdf15/P150047a.pdf.

[154]. European Medicines Agency. Tagrisso: public assessment report — product information.European Medicines Agency, 2017 EMA 2016 21 October 2017, Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR__Product_Information/human/004124/WC500202022.pdf.

[155]. Qiagen. Therascreen EGFR Plasma RGQ PCR Kit Handbook — Version 2.Qiagen, 2014 21 October 2017 Available from: https://www.qiagen.com/at/resources/resourcedetail?id=d217c9ca-b9f6-4891-8c46−32b71e83d88a&lang=en.

[156]. Remon J, et al. Liquid biopsies for molecular profiling of mutations in non-small cell lung cancer (NSCLC) patients lacking tissue samples.Inivata 2016; Available from: http://inivata.com/wp-content/uploads/2016/09/MAP2016posterFINAL.pdf.

[157]. US National Library of Medicine Next generation pErsonalized tX(Therapy) with plasma DNA trial-2 in refractory solid tumors (The NEXT-2 Trial).ClinicalTrials.gov, 2015.

[158]. Talasaz A, et al. Ultra-high quality sequencing assay for comprehensive genetic panel analysis of rare tumor-derived circulating cell-free DNA. Boston: ASHG; 2013.

[159]. US National Library of Medicine. Monitoring plasma tumor dna in early-stage breast cancer.ClinicalTrials.gov 2016 24 October 2017 Available from: https://clinicaltrials.gov/ct2/show/NCT02743910.

[160]. Gormally E, et al. TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: a prospective study. Cancer Res 2006;66(13):6871. [PubMed: 16818665]

[161]. Phallen J, et al. Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl Med 2017;9(403).

[162]. Chan KCA, et al. Analysis of plasma epstein–barr virus DNA to screen for nasopharyngeal cancer. New Eng J Med 2017;377(6):513–22. [PubMed: 28792880]

[163]. Cheng SH, et al. Noninvasive prenatal testing by nanopore sequencing of maternal plasma DNA: feasibility assessment. Clin Chem 2015;61(10):1305. [PubMed: 26286915]

[164]. Norris AL, et al. Nanopore sequencing detects structural variants in cancer. Cancer Biol Ther 2016;17(3):246–53. [PubMed: 26787508]

[165]. Burnham P, et al. Single-stranded DNA library preparation uncovers the origin and diversity of ultrashort cell-free DNA in plasma. Sci Rep 2016;6:27859. [PubMed: 27297799]

[166]. Wu DC, Lambowitz AM.Facile single-stranded DNA sequencing of human plasma DNA via thermostable group II intron reverse transcriptase template switching. Sci Rep 2017;7:8421. [PubMed: 28827600]

[167]. Mouliere F, et al. Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. Mol Oncol 2014;8(5):927–41. [PubMed: 24698732]

[168]. Cheng THT, et al. Genomewide bisulfite sequencing reveals the origin and time-dependent fragmentation of urinary cfDNA. Clin Biochem 2017.

[169]. Atamaniuk J, et al. Increased concentrations of cell-free plasma DNA after exhaustive exercise. Clin Chem 2004;50(9):1668. [PubMed: 15331502]

[170]. Fatouros IG, et al. Cell-free plasma DNA as a novel marker of aseptic inflammation severity related to exercise overtraining. Clin Chem 2006;52(9):1820–4. [PubMed: 16840584]

[171]. Gormally E, et al. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. Mutat Res 2007;635(2–3):105–17. [PubMed: 17257890]

[172]. Wan JCM, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;17(4):223–38. [PubMed: 28233803]

[173]. Yu S, et al. Tumor-derived exosomes in cancer progression and treatment failure. Oncotarget 2015;6(35):37151–68. [PubMed: 26452221]

[174]. Kahlert C, Kalluri R.Exosomes in tumor microenvironment influence cancer progression and metastasis. J Mol Med (Berl) 2013;91(4):431–7. [PubMed: 23519402]

[175]. Yang S, et al. Detection of mutant KRAS and TP53 DNA in circulating exosomes from healthy individuals and patients with pancreatic cancer. Cancer Biol Ther 2017;18(3):158–65. [PubMed: 28121262]

[176]. Kahlert C, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. J Biolo Chem 2014;289(7):3869–75.

[177]. Thakur BK, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res 2014;24(6):766–9. [PubMed: 24710597]

[178]. Sullivan RJ, et al. Plasma-based monitoring of BRAF mutations during therapy for malignant melanoma (MM) using combined exosomal RNA and cell-free DNA analysis. J Clin Oncol 2015;33(15_suppl) pp. 9017–9017.

[179]. Shao H, et al. Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. Nat Commun 2015;6:6999. [PubMed: 25959588]

[180]. Huang X, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC Genom 2013;14:319.

[181]. Li M, et al. Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. Philos Trans R Soc B: Biol Sci 2014;369(1652):20130502.

[182]. Lunavat TR, et al. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells–Evidence of unique microRNA cargos. RNA Biol 2015;12(8):810–23. [PubMed: 26176991]

[183]. Matullo G, Naccarati A, Pardini B.MicroRNA expression profiling in bladder cancer: the challenge of next-generation sequencing in tissues and biofluids. Int J Cancer 2016;138(10): 2334–45. [PubMed: 26489968]

[184]. Qin Y, et al. High-throughput sequencing of human plasma RNA by using thermostable group II intron reverse transcriptases. RNA 2016;22(1):111–28. [PubMed: 26554030]

[185]. Terrin L, et al. Relationship between tumor and plasma levels of hTERT mRNA in patients with colorectal cancer: implications for monitoring of neoplastic disease. Clin Cancer Res 2008;14(22):7444–51. [PubMed: 19010861]

[186]. Pucciarelli S, et al. Telomere-specific reverse transcriptase (hTERT) and cell-free RNA in plasma as predictors of pathologic tumor response in rectal cancer patients receiving neoadjuvant chemoradiotherapy. Ann Surg Oncol 2012;19(9):3089–96. [PubMed: 22395986]

[187]. Ng EK, et al. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. Clin Chem 2002;48(8):1212–17. [PubMed: 12142376]

[188]. Nottingham RM, et al. RNA-seq of human reference RNA samples using a thermostable group II intron reverse transcriptase. RNA 2016;22(4):597–613. [PubMed: 26826130]

[189]. Burgos KL, Van Keuren-Jensen K.RNA isolation for small RNA next-generation sequencing from acellular biofluids. Methods Mol Biol 2014;1182:83–92. [PubMed: 25055903]

[190]. Yeri A, et al. Total extracellular small RNA profiles from plasma, saliva, and urine of healthy subjects. Scientific Reports 2017;7:44061. [PubMed: 28303895]

[191]. Khagi Y, et al. Hypermutated circulating tumor DNA: correlation with response to checkpoint inhibitor–based immunotherapy. Clin Cancer Res 2017;23(19):5729. [PubMed: 28972084]

Table 1

Comparison of PCR-based approaches and next-generation sequencing for their use in cfDNA analysis and applications.

Methods	PCR-based approaches	Next-generation sequencing
Features	Highly sensitive (MAF 0.001%)	Less sensitive (MAF 0.1–10%)
	Fast to run	Long to run
	Limited multiplexing	High levels of multiplexing
	Easy to interpret results	Requires bioinformatics and computational resources
	Low cost	High cost
	Absolute quantification	Approximate quantification
Applications	Identification of single or few mutations for molecular diagnostics and guiding therapy	Identification of multiple mutations for molecular diagnosis and guiding therapy
	Tracking mutations during therapy	Tracking mutations during therapy
	Limited to mutations in assay	Discovery of new mutations
	Measuring mutation load to predict prognosis	Measuring mutation load to predict prognosis
	Monitoring for minimal residual disease	Monitoring for minimal residual disease requires UMIs and sequencing to redundancy
	Copy number alteration detection in single or few genes	Broad copy number alteration detection across whole genome
	Studies of emerging resistance with well-defined mechanisms	Studies of all emerging resistance
	Tracking recurrence of known mutations	Tracking recurrence of known or unknown mutations

Abbreviations: PCR polymerase chain reaction, MAF mutant allele fraction, UMI unique molecular indexes, cfDNA cell-free DNA.