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REVIEW

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CtDNA: What We Know and What We Are Looking For

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Abstract
In recent years, circulating tumor DNA (ctDNA) has become an essential analyte in both scientific research and the treatment of oncology patients. While many questions remain unanswered, the information contained in ctDNA molecules is crucial for understanding the biological characteristics of malignant tumors. Currently, plasma is the standard biological source of ctDNA, although other sources are being explored as potential alternatives. The kinetics of ctDNA is influenced by various factors, all of which must be considered when determining the timing and volume of body fluid samples. CtDNA has applications in oncology, particularly in selecting the most appropriate targeted therapy based on the mutations present in tumors. Its role in the early detection of cancer or minimal residual disease is still under investigation.
Our aim was to highlight outstanding challenges for the applications of ctDNA in research and molecular diagnostics. To expand the use of ctDNA in clinical practice it is necessary to establish clear and standardized protocols for the isolation and detection of ctDNA. Until then, we must continue to summarize existing literature, highlighting the issues that we are eager to resolve.

Introduction

As our understanding of the biology of malignant tumors grows, it is increasingly evident that significant inter- and intra-tumor heterogeneity poses a major challenge in treating cancer patients. The advancement of therapeutic strategies and the more frequent customization of treatment pathways for individual patients have led to the development of personalized treatment approaches (1). Such treatments require ongoing monitoring to predict therapy response, the development of resistance, disease progression, and potential side effects. Ideally, valuable information can be obtained from a tumor biopsy. However, the tumor's location and the patient's overall health often make rebiopsy at various time points impractical. This challenge has driven the intensive development of liquid biopsy methods aimed at identifying suitable biological markers in a more accessible and less invasive manner. Components of the tumor and its microenvironment can be isolated from body fluids, including circulating tumor cells (CTCs), extracellular vesicles, tumor-educated platelets, cell-free RNA (cfRNA), and circulating tumor DNA (ctDNA) (2, 3).
The presence of circulating fragments of DNA was first described in 1948, although significant findings were scarce until 1977 (4). At that point, Leon et al. reported that patients with various malignancies exhibited increased levels of circulating free cellular DNA (cfDNA) compared to healthy individuals (5). In 1989, Strawn et al. concluded that the elevated cfDNA levels in cancer patients were due to DNA fragments released into circulation by cancer cells (6). Today, we recognize that ctDNA, which originates specifically from malignant cells, constitutes a fraction of the total cfDNA found in body fluids.

The insights provided by ctDNA have made it an essential analyte in cancer treatment. This review aims to summarize the current literature on ctDNA, identify the associated challenges and issues, and predict the future directions for research in this field.

What is ctDNA?

Several mechanisms contribute to the release of ctDNA. Most commonly, it results from tumor cell death, but it can also arise from cellular senescence, the active secretion of extracellular vesicles, or the egestion of mitochondrial DNA (2).

CfDNA consists of fragments typically ~167 base pairs (bp) in size, which corresponds with nucleosome-associated fragments of DNA, primarily originating from hematopoietic cells. (2, 7). However, it is believed that cells from other organ systems may also release cfDNA under both physiological and pathological conditions, which warrants further investigation (7). The size of ctDNA fragments can vary significantly, depending on the biological source from which the ctDNA is isolated; generally, ctDNA is smaller than 150 bp (8). It is hypothesized that cfDNA mainly comes from cells undergoing apoptosis, which are subsequently phagocytosed by macrophages via apoptotic blebs, leading to their release into circulation. The role of necrosis in the release of total cfDNA is still not fully understood (9).

In patients with malignancies, particularly in advanced disease stages, the amount of cfDNA tends to increase. It is assumed that this increase in cfDNA, specifically ctDNA, is largely derived from necrotic malignant cells. Additionally, autophagy may play a critical role, serving as an alternative energy source for tumors (10). Regardless of whether the mechanism of cell death is apoptosis, necrosis, or another type, ctDNA is also phagocytosed by immune cells and subsequently released as smaller fragments into the tumor microenvironment or circulation. It is also important to note that ctDNA can originate from living tumor cells through their extracellular vesicles (2).

The levels of cfDNA and ctDNA in circulation depend directly on the balance between the release of DNA fragments and their clearance. Studies have shown that the half-life of cfDNA ranges from 16 minutes to 2.5 hours (2). The breakdown and excretion of DNA fragments involve mechanisms such as DNases present in circulation, active clearance of nucleosomes, and filtration through organs like the kidneys and lymph nodes (11). The association of ctDNA with macromolecular complexes, as well as its size, also influences the rate of clearance (12). Further research is needed to better understand the kinetics of ctDNA.

Moreover, a significant challenge remains in distinguishing ctDNA from cfDNA. Current studies are focusing on three main approaches to analyze the structure of ctDNA: examining tail motifs, nucleosome positioning, and methylation patterns (13). By enhancing these techniques, researchers strive to establish methods for quantifying ctDNA, a challenge that remains unsolved. Thus far, ctDNA levels are often described numerically through variant allele frequency (VAF), which is the only way to quantify ctDNA within the total cfDNA present (2).

What Affects ctDNA Levels?

Several important factors influence the levels of ctDNA in the body beyond its kinetic characteristics.

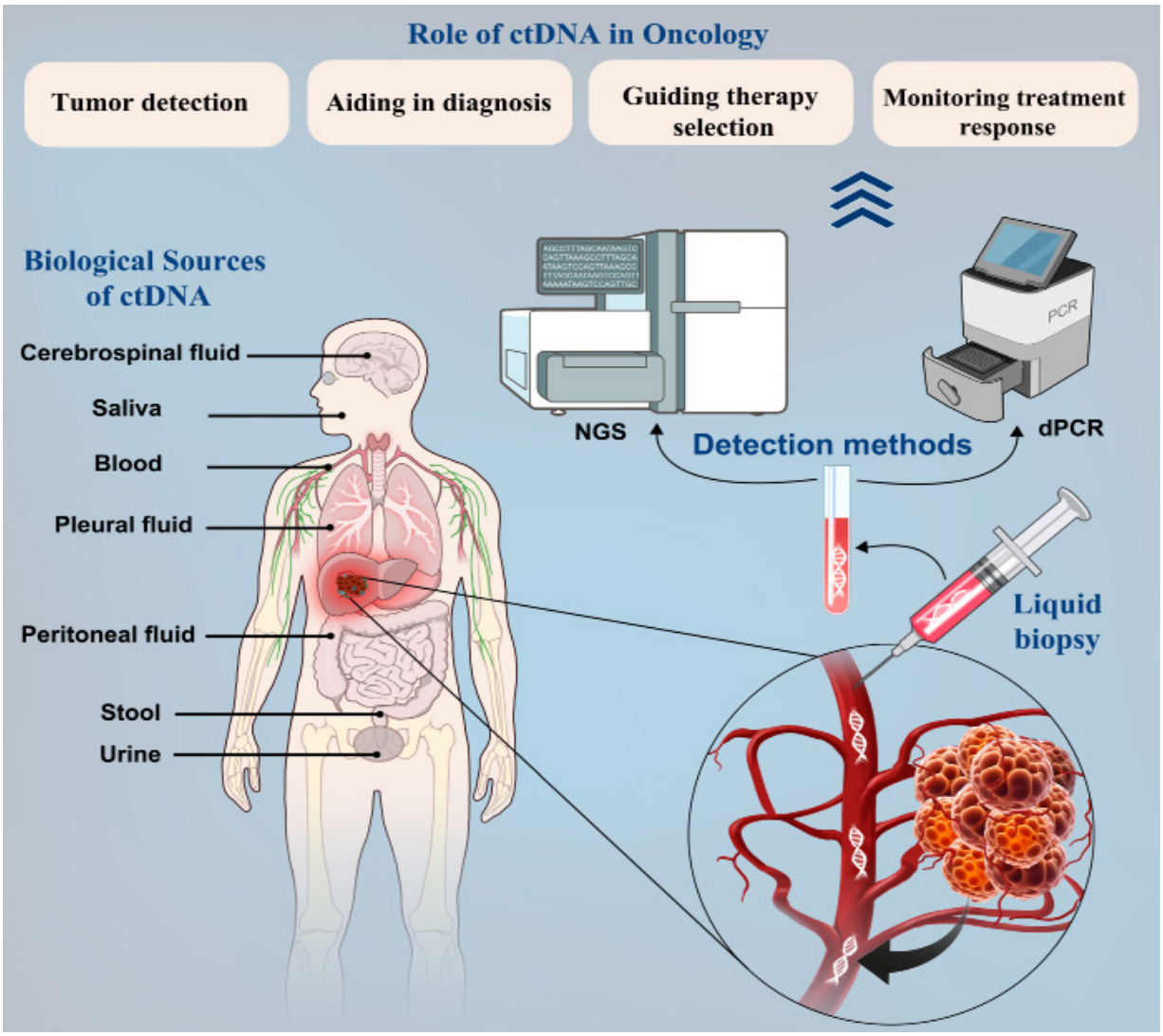
Currently, it is understood that the level of ctDNA primarily depends on the type of malignant tumor present. For instance, tumors located in the central nervous system (CNS) tend to release undetectable amounts of ctDNA into the bloodstream (14). This is why it is crucial to consider the biological source from which ctDNA is isolated, tailoring this approach to the specific type of tumor and its stage of progression. In addition to CNS tumors, low levels of ctDNA are also observed in conditions such as medulloblastoma, kidney cancer, prostate cancer, and thyroid cancer. Conversely, detecting ctDNA is significantly more straightforward in cancers of the ovary, liver, pancreas, bladder, lung, stomach, breast, and colon, among others (14). Furthermore, the histological characteristics of the tumor are also important. Research indicates that cancers with a pronounced necrotic profile release larger amounts of ctDNA. This phenomenon is particularly evident in squamous cell lung cancer and triple-negative breast cancer. These findings further emphasize the role of necrosis in influencing ctDNA levels (15, 16).

The level of ctDNA is influenced by the stage of the disease. Numerous studies have demonstrated that patients with more advanced stages of the disease have significantly higher amounts of ctDNA. For example, in the early stages of non-small cell lung cancer (NSCLC), the ctDNA constitutes only 0.01% of cfDNA, whereas in the later stages, this percentage can rise to as much as 10% (14, 17).

Research has also shown a strong correlation between tumor size and volume with ctDNA levels. Patients with larger tumors tend to have a significantly higher VAF value (18).

When sampling, detecting, and analyzing ctDNA, it is crucial to consider the impact of therapy on these measurements. After surgical removal of a tumor, the ctDNA level in the body drops significantly. However, tissue damage caused during the procedure can lead to a substantial increase in total cfDNA levels immediately afterward. This surge in

cfDNA may mask the presence of ctDNA, potentially leading to false negative results that suggest the disease is absent when it may not be (19). Additionally, treatments like radiotherapy and chemotherapy, which have cytotoxic effects, can further elevate cfDNA levels, diminishing the detectability of ctDNA. To avoid receiving misleading negative results, it is essential to collect cfDNA samples at carefully chosen time points, considering the clearance rate of DNA fragments (2, 19).



The primary biological sources for isolating circulating tumor DNA (ctDNA) have been identified. After detection using next-generation sequencing (NGS) or digital polymerase chain reaction (dPCR) methods, ctDNA plays a role in various stages of personalized treatment for oncology patients.

What Are the Biological Sources of ctDNA?

Currently, ctDNA isolated from plasma has the most significant application in clinical practice. Plasma is generally a better biological source than serum due to the smaller amounts of cfDNA found in serum (2, 3). However, as mentioned in the previous chapter, certain tumors release ctDNA in insufficient quantities into the bloodstream, complicating the detection process. Consequently, recent research has focused on exploring other biological sources within the body from which ctDNA can be isolated. These alternative sources often yield a significantly higher proportion of ctDNA compared to the total cfDNA present, particularly due to the reduced number of the hematopoietic system cells. In some instances, ctDNA from these sources may more accurately reflect the clonal heterogeneity of the tumor itself than ctDNA obtained from plasma (8).

Urinary ctDNA can originate from plasma that has undergone glomerular filtration or from tumor cells located directly within the urinary tract (20, 21). Isolating ctDNA from urine presents several advantages: it is readily accessible and convenient for patients. Additionally, the level of cfDNA in urine is significantly lower than in plasma because there is no lysis of blood cells, although cfDNA from epithelial cells in the urinary tract may still be present (8). However,

a challenge with urine as a biological source is the variability in the size of the ctDNA molecules due to glomerular filtration. Furthermore, the larger volume of urine samples compared to plasma samples can pose difficulties in the isolation process, while also leading to excessive dilution of ctDNA. The amount of ctDNA found in urine can fluctuate throughout the day, especially if the patient is undergoing systemic anticancer therapy. There are also concerns regarding the optimal methods for storing and transporting urine samples, as well as the ideal time frames for analysis, given the short half-life of insufficiently protected ctDNA (22, 23). Despite these challenges, research has demonstrated that urinary ctDNA provides valuable information about mutations in the *EGFR* and *KRAS* genes in patients with NSCLC and enhances the accuracy of results when combined with plasma ctDNA analysis (24). Promising results have also been reported in the context of breast cancer and predicting relapses in hepatocellular carcinoma (8). Undoubtedly, ctDNA isolated from urine offers crucial insights regarding malignant tumors of the urinary system.

For tumors located in CNS, which typically do not release ctDNA into the bloodstream, *cerebrospinal fluid* (CSF) can serve as an alternative source for diagnosis and predicting disease relapse. CSF contains a lower level of circulating immune cells compared to blood, leading to lower levels of cfDNA (8). CtDNA can provide genetic information about primary CNS cancers or metastases from distant sites, such as melanoma. In the case of gliomas, several molecular markers are well-established. Their detection in ctDNA using a digital polymerase chain reaction (dPCR) approach allows for the classification of tumors into different molecular subtypes, positively influencing the choice of optimal therapy (25, 26). However, CSF is less readily available than other biological sources and its sampling is associated with high risks, making routine or repeated sampling nearly impossible. Further research is needed to standardize the processes for isolating and storing CSF, and this research is complicated by the necessity of obtaining special ethical approvals (8).

Pleural and peritoneal fluids are in close contact with tumors and may contain ctDNA even when plasma results are negative. The overall levels of cfDNA in these fluids are lower than in blood since they generally lack blood cells. These fluids have been utilized in clinical analyses for NSCLC and colon cancer. Studies have indicated that target mutations in pleural and peritoneal effusion can be detected with higher VAFs than those detectable in plasma (27, 28). However, the sampling procedures for these fluids can be invasive and are only pursued when they offer significant benefits for the patient, which complicates their use in monitoring oncology patients.

Other biological sources for ctDNA isolation, such as *saliva* and *stool*, have shown potential as well. While salivary ctDNA provides useful information concerning malignant tumors of the head and neck, the size of the DNA fragments, which measures 40-60 bp, complicates analysis (29). In stool samples, human DNA constitutes only 0.01% of the total, as the sample is predominately composed of the gastrointestinal microbiome, complicating the isolation and detection of ctDNA (30). Ongoing research will likely reveal the importance of additional biological sources for ctDNA, including *seminal fluid*, *bile*, *uterine lavage fluid*, *vaginal fluid*, and more (8).

What are the methods for isolation and detection of ctDNA?

Research has focused significant attention on the development of techniques for the isolation and detection of ctDNA in recent years, alongside advancing knowledge about ctDNA and liquid biopsies in general. CtDNA represents a portion of the total cfDNA regardless of the biological source from which it is isolated.

Currently, isolation methods have been standardized primarily for blood or plasma samples. When using standard tubes with K2 EDTA, the entire analysis must be completed within six hours of blood collection, with careful maintenance of the cold chain during transport. Alternatively, specialized collection tubes with stabilizing agents that can keep cfDNA stable at room temperature for up to 14 days can be used (31). Effective isolation requires the complete removal of all cellular components from the sample. For this reason, it is recommended to perform a two-step centrifugation at 1600g for 10 minutes when isolating plasma. To ensure the integrity of cfDNA during storage, samples should ideally be kept at -80°C and should avoid multiple freeze-thaw cycles (32).

There are three primary techniques used for isolation: phase separation, silica membrane-based isolation, and magnetic bead-based isolation. The chosen isolation method is crucial for the success of the subsequent detection, as the quantity and preservation of ctDNA fragments depend heavily on it (2).

The initial platform for ctDNA detection was quantitative polymerase chain reaction (qPCR). However, due to its reduced sensitivity, dPCR and next-generation sequencing (NGS) have gained prominence. While dPCR offers high sensitivity for detection, it is limited by the finite number of mutations that can be analyzed in a single assay. One advantage of dPCR over NGS is that it does not require bioinformatic analysis, allowing for faster results, which is beneficial for patients (3).

Nevertheless, improvements in NGS, such as the incorporation of molecular tags (barcoding) for individual DNA molecules and advancements in bioinformatic analyses, have addressed many of the previous limitations, making NGS increasingly user-friendly. Today, NGS provides comprehensive information regarding the identification of genetic

alterations, making it an indispensable method for ctDNA analysis and detection (3, 33).

What is the Role of ctDNA in Oncology?

Circulating ctDNA is emerging as a valuable analyte in oncology, offering potential for early disease detection, aiding in diagnosis, guiding therapy selection, and monitoring treatment response. It may also play a role in predicting resistance or relapse. Currently, ctDNA is primarily utilized to select the most appropriate therapy for patients based on target mutations identified within the ctDNA, which represents the tumor's complete genetic landscape. This is in contrast to traditional biopsy samples, which usually represent only a small fraction of the tumor.

The first major approval for the clinical use of ctDNA was the Cobas® EGFR Mutation test for detecting mutations in the *EGFR* gene (specifically, exon 19 deletions and the exon 21 L858R mutation) using the qPCR method.This allows for the identification of patients eligible for tyrosine kinase inhibitor therapy (34). Additionally, a kit for detecting 11 *PIK3CA* mutations in ctDNA through PCR has been used to select breast cancer patients for treatment with alpesilib (3, 34). As the list of targetable mutations has expanded to include the exon 20 T790M mutation in the *EGFR* gene, *KRAS* G12C, *ALK*, and *MET* exon 14 single-nucleotide variants (SNVs), the detection methods have advanced as well. This led to the development of the first gene panels for NGS testing. Today, there are NGS panels available for various cancers, including NSCLC and panels for prostate, breast, and ovarian cancers that also test for *BRCA1* and *BRCA2* mutations (34).

One notable limitation of ctDNA is its low concentration during the early stages of cancer, which can hinder early detection. Research has explored the use of ctDNA for the early detection of urothelial cancers from urine samples and pancreatic cancer from stool samples (8). These alternative biological samples were chosen under the assumption that they would contain higher levels of ctDNA compared to blood samples. Furthermore, a study evaluated whether a targeted methylation-based test could be used for screening multiple cancers in individuals over 50 years of age. While the test yielded promising results, it also resulted in 57 false positives, along with reduced sensitivity in detecting early-stage disease (35).

Moreover, ctDNA can indicate the presence of minimal residual disease after surgical intervention or completion of therapy. It can also be used to monitor the patient's response to treatment and to detect early resistance before it becomes apparent through standard diagnostic methods. Currently, the most significant applications of ctDNA monitoring are found in clinical studies (2, 34). These studies define specific time points to monitor ctDNA levels before and after the administration of neoadjuvant or adjuvant therapy. From this data, it is possible to predict the development of resistance to the treatment or the likelihood of disease relapse (34). The combination of these findings with research from other studies will pave the way for more widespread use of ctDNA in patient monitoring throughout the treatment process.

Conclusion

Based on the current literature and the findings summarized in our review paper, we can conclude that two key challenges must be addressed for ctDNA to be widely used in clinical practice.

The first challenge is to gain a better understanding of the kinetics of ctDNA. This involves defining the characteristics of different malignant tumors and identifying the factors that influence the release of ctDNA into the bloodstream. This knowledge will help in selecting the most appropriate biological source for each type of cancer. Additionally, when determining the timing and quantity of sample collection, it is crucial to consider all factors that may impact ctDNA kinetics, such as surgical interventions and cytotoxic therapies. Establishing a protocol for ctDNA sampling can help minimize the occurrence of false-negative results due to a low proportion of ctDNA in the total cfDNA.

The second challenge is the standardization of methods for isolating and detecting ctDNA from various biological sources, as well as improving technology to enhance sensitivity. This advancement will allow for the detection and analysis of ctDNA present in small quantities.

Research in the coming years will provide answers to these challenges. It is clear that ctDNA holds the potential to significantly improve the personalized treatment of oncology patients, potentially making many malignant tumors manageable and even curable, even in the later stages of the disease.

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References:

1. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature 2013; 501(7467):338-45

2. Sánchez-Herrero E , Serna-Blasco R, Robado de Lope L, González-Rumayor V , Romero A , Provencio M. Circulating Tumor DNA as a Cancer Biomarker: An Overview of Biological Features and Factors That may Impact on ctDNA Analysis. Front Oncol. 2022 Jul 20:12:943253.

3. Dang DK, Park BH. Circulating tumor DNA: current challenges for clinical utility. J Clin Invest. 2022 Jun 15;132(12):e154941.

4. Mendel P, Metais P. [Nuclear Acids In Human Blood Plasma] C R Seances Soc Biol Fil. 1948 Feb;142(3-4):241-3.

5. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977 Mar;37(3):646-50.

6. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology. 1989;46(5):318-22.

7. Kustanovich A, Schwartz R, Peretz T, Grinshpun A. Life and death of circulating cell-free DNA. Cancer Biol Ther. 2019;20(8):1057-1067.

8. Tivey A, Church M, Rothwell D, Dive C, Cook N. Circulating tumour DNA - looking beyond the blood. Nat Rev Clin Oncol. 2022 Sep;19(9):600-612.

9. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 1998 Jan 1;391(6662):43-50.

10. Sato K, Tsuchihara K, Fujii S, Sugiyama M, Goya T, Atomi Y, Ueno T, Ochiai A, Esumi H. Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. Cancer Res. 2007 Oct 15;67(20):9677-84.

11. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA, Kinzler KW, Vogelstein B, Diaz Jr LA. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008 Sep;14(9):985-90.

12. Mittra I, Nair NK, Mishra PK. Nucleic acids in circulation: are they harmful to the host? J Biosci. 2012 Jun;37(2):301-12.

13. Barefoot ME, Loyfer N, Kiliti AJ, McDeed 4th AP, Kaplan T, Wellstein A. Detection of Cell Types Contributing to Cancer From Circulating, Cell-Free Methylated DNA. Front Genet. 2021 Jul 27:12:671057.

14. Bettegowda C, Sausen M, Leary RJ et al. Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies. Sci Transl Med. 2014 Feb 19;6(224):224ra24.

15. Zhang B, Niu X, Zhang Q et al. Circulating tumor DNA detection is correlated to histologic types in patients with early-stage non-small-cell lung cancer. Lung Cancer. 2019 Aug:134:108-116.

16. Livasy CA , Karaca G, Nanda R, Tretiakova MS, Olopade O, Moore DT, Perou CM. Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol. 2006 Feb;19(2):264-71.

17. Chabon JJ, Hamilton EG, Kurtz DM. Integrating genomic features for non-invasive early lung cancer detection. Nature. 2020 Apr;580(7802):245-251.

18. Abbosh C, Birkbak NJ, Wilson GA et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. Nature. 2017 Apr 26;545(7655):446-451.

19. Szpechcinski A, Chorostowska-Wynimko J, Kupis W, Maszkowska-Kopij K, Dancewicz M, Kowalewski J, Orlowski T. Quantitative analysis of free-circulating DNA in plasma of patients with resectable NSCLC. Expert Opin Biol

Ther. 2012 Jun:12 Suppl 1:S3-9.

20. Melkonyan HS, Feaver WJ, Meyer E, Scheinker V, Shekhtman EM, Xin Z, Umansky SR. Transrenal nucleic acids: from proof of principle to clinical tests. Ann N Y Acad Sci. 2008 Aug:1137:73-81.

21. Green EA, Li R, Albiges L, Choueiri TK, Freedman M, Pal S, Dyrskjøt S, Kamat AM. Clinical Utility of Cell-free and Circulating Tumor DNA in Kidney and Bladder Cancer: A Critical Review of Current Literature. Eur Urol Oncol. 2021 Dec;4(6):893-903.

22. Augustus E, Casteren KV, Sorber L et al. The art of obtaining a high yield of cell-free DNA from urine. PLoS One. 2020 Apr 6;15(4):e0231058.

23. Yao W, Mei C, Nan X, Hui L. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. Gene. 2016 Sep 15;590(1):142-8.

24. Reckamp KL, Melnikova VO, Karlovich C et al. A Highly Sensitive and Quantitative Test Platform for Detection of NSCLC EGFR Mutations in Urine and Plasma. J Thorac Oncol. 2016 Oct;11(10):1690-700.

25. Wesseling P, Capper D . WHO 2016 Classification of gliomas. Neuropathol Appl Neurobiol. 2018 Feb;44(2):139-150.

26. Fujioka Y, Hata N, Akagi Y et al. Molecular diagnosis of diffuse glioma using a chip-based digital PCR system to analyze IDH, TERT, and H3 mutations in the cerebrospinal fluid. J Neurooncol. 2021 Mar;152(1):47-54.

27. Durin L, Pradines A , Basset C, Ulrich B, Keller L, Dongay V, Favre G, Mazieres J, Guibert N. Liquid Biopsy of Non-Plasma Body Fluids in Non-Small Cell Lung Cancer: Look Closer to the Tumor! Cells. 2020 Nov 16;9(11):2486.

28. Zho Z et al. Lung cancer genomic alterations in cell free DNA in pleural effusion compared to plasma. J. Clin. Oncol. 2017; 35(15):e23206.

29. Wei F, Chien-Chung Lin, Joon A et al. Noninvasive saliva-based EGFR gene mutation detection in patients with lung cancer. Am J Respir Crit Care Med. 2014 Nov 15;190(10):1117-26.

30. Klaassen CKW, Jeunink MAF, Prinsen CFM, Ruers TJM, Tan AC, Strobbe LJA, Thunnissen FB. Quantification of human DNA in feces as a diagnostic test for the presence of colorectal cancer. Clin Chem. 2003 Jul;49(7):1185-7.

31. Fernando MR, Chen K, Norton S, Krzyzanowski G, Bourne D, Hunsley B, Ryan WL, Bassett C. A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. Prenat Diagn. 2010 May;30(5):418-24.

32. Chan AKC, Sze-Wan Yeung, Wing-Bong Lui, Rainer TH, Lo DYM. Effects of preanalytical factors on the molecular size of cell-free DNA in blood. Clin Chem. 2005 Apr;51(4):781-4.

33. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011 Jun 7;108(23):9530-5.

34. Vellanki PJ, Ghosh S, Pathak A et al. Regulatory implications of ctDNA in immuno-oncology for solid tumors. J Immunother Cancer. 2023 Feb;11(2):e005344.

35. Turning the tide of early cancer detection. Nat Med. 2024 May;30(5):1217.