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Circulating Tumor DNA Analysis: Practical Laboratory Insights for Clinicians.

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Abstract:

Liquid biopsies provide a non-invasive way to assess cancer patients. One of the most popular and promising sources is circulating cell-free DNA (ccfDNA). Early cancer identification, treatment response evaluation, prognosis, and prognostic assessments can all benefit from the detection of tumor DNA among ccfDNA (ctDNA). Clinicians and laboratory scientists work together to provide analyses that can improve patient care. Similar to how laboratory scientists should be aware of the clinical requirements in order to deliver pertinent analyses, it is crucial that doctors understand the fundamental ideas and analyses underlying ctDNA results as their application approaches clinical practice. In this Perspective, we outline the entire ctDNA analysis process, from preanalytical standards to reporting and analysis of results, and we point out several crucial aspects that must be taken into consideration when applying them to clinical practice.

Keywords: Tumor DNA, Laboratory, Analysis, Clinicians, Circulating Tumor.

Introduction:

A more individualized approach to cancer patient diagnosis, treatment, and monitoring has been made possible by an increasing awareness of the intrinsic molecular and genetic profiles of

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various tumor forms. The lack of very specific molecular indicators, the sensitivity and specificity of imaging methods, and the general lack of clinical signs make cancer diagnosis challenging, particularly in the early stages. Cancer patients are typically treated and monitored using a multimodal strategy that includes tissue samples, radiological findings, patient consultations, and biochemical marker analysis (e.g., carcinoembryonic antigen (CEA), prostate-specific antigen (PSA) (J He et al., 2023).

Although customized medicine has the potential to substantially increase cancer survival rates, its application in clinical practice necessitates more precise and sensitive instruments for early cancer identification and ongoing therapy monitoring. Tumor-derived circulating cell-free DNA (ctDNA) has the potential to significantly enhance the use of biomarkers in a customized medical context and offers an intriguing route for a more customized approach to cancer management. A multimodal strategy using both ctDNA and radiological and clinical results has been used in a number of studies for both prognostic and diagnostic purposes (MJM Magbanua et al., 2021).

A number of unique characteristics set circulating cell-free DNA (ccfDNA) from healthy tissue apart from ctDNA, which is fragmented DNA produced or shed from cancer cells into the bloodstream. These include alterations in DNA methylation, copy number aberrations, genomic rearrangements, and mutations of oncogenes and tumor suppressor genes. Additionally, ctDNA is around 150 bp shorter than ccfDNA, which is roughly 160 to 170 bp. The most popular methods for measuring ctDNA are the identification of mutations and modifications in methylation patterns. Tumor-agnostic analysis is the process of detecting ctDNA without first profiling the tumor. When genetic profiling of the tumor is carried out to identify one or more targets that can be monitored in liquid biopsies, a tumor-informed strategy can be employed (L Keller, 2021).

Early cancer detection, risk stratification, genomic profiling for treatment selection, prognosis, minimal residual disease (MRD) detection, and treatment resistance monitoring are just a few of the many possible uses of ctDNA detection. When blood samples are taken over time, ctDNA analysis has tremendous potential to guide treatment and track mutational and epigenetic changes in the tumor due to its short half-life, which ranges from minutes to one to two hours (F Mouliere et al., 2018).

The primary factors influencing ctDNA measurement are discussed in this perspective, along with the difficulties in identifying biomarkers in cancer patients' plasma. We outline the two primary methods for ctDNA detection and discuss the major problems in the preanalytical stages.

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Preanalytical Factors for ctDNA Detection in Cancer Patients:

The low fraction of ctDNA in circulation compared to noncancer ccfDNA is the primary obstacle to the clinical utility of ctDNA analysis. Highly sensitive methods are utilized to find ctDNA in samples, which calls for a rigorous quality control program to guarantee assay precision, accuracy, and repeatability. Strict quality control is much more necessary for sequential sample analyses so that the results may be compared. Because of these issues, preanalytical factors and sample material quality must be carefully considered before the analytical stage. By taking part in external quality assurance programs and using reference materials to track the results of laboratory testing, standardization of the preanalytical phase may be justified (EA Klein et al., 2021).

Selection of Sample Material:

Tissue biopsies continue to be the gold standard for cancer diagnosis and molecular characterization of solid tumors; however, tumor sampling is not always feasible and is not appropriate for frequent monitoring, making liquid biopsies an appealing complementary material. Although other biospecimens are used for ctDNA detection, blood is the most frequently used type of liquid biopsy. Other specimens include urine, pleural effusion, and ascites (J Tie et al., 2022).

Both serum and plasma can be used to extract ccfDNA, with serum samples showing larger amounts. High molecular weight (HMW) DNA from leukocyte lysis has been identified as the cause of the elevated ccfDNA content. Plasma samples are superior in terms of mutation detection efficiency and reproducibility, according to several studies comparing ctDNA detection in serum and plasma (M García et al., 2023).

To find uncommon occurrences with the same LOD, more plasma should be utilized. There are no guidelines for the exact volume in the various therapeutic contexts, and practical factors (such as the patient's frailty or concurrent blood draws for other tests) will frequently have an impact.

Sample Material Handling:

Plasma sample storage and transportation should be carefully evaluated because improper methods can have a significant impact on ctDNA findings. The possibility of triggering leukocyte lysis is especially concerning since the release of HMW DNA will make it more difficult to identify ctDNA. The concentration and detection of ctDNA can be impacted by several factors, including centrifugation, pipetting methods, storage tubes, storage temperature, and mechanical stress during handling (J Tie et al., 2016). The duration of storage prior to plasma separation influences the selection of blood collection tubes. Because they are inexpensive and have DNase-inhibiting qualities, EDTA blood collection tubes are ideal for drawing blood, but they must be processed within three to six hours to prevent an increase in

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HMW DNA content. Specialized blood collection tubes with preservative chemicals that prevent leukocyte lysis should be utilized for storage longer than six hours (e.g., Streck Cell-Free DNA BCT, PAXgene Blood cfDNA Tube, Roche Cell-free DNA Collection Tube). All of these tubes can be delivered and kept at room temperature for at least seven days (D Kotani et al., 2023).

The content and integrity of both ctDNA and HMW DNA can be impacted by mechanical stress during blood sample transportation and agitation, therefore samples should be handled carefully. When samples are delivered, some of these impacts can be lessened by using specific tubes (TV Henriksen et al., 2023).

Sample Quality Control:

The effectiveness of the ccfDNA extraction, the concentration after extraction, fragment length, contamination with HMW DNA, and the ratio of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA) are some of the variables that may be pertinent to consider during quality control. Before findings are announced, all laboratories using ctDNA should establish acceptable values for quality control variables (FC Bidard et al., 2022).

Extraction Effectiveness:

Because the amount of ctDNA in samples is limited, effective ccfDNA extraction is an essential step. There are a number of commercial extraction kits available, and many articles have compared them. While some kits are biased toward the isolation of short fragments, others promote high DNA yield. Automated extraction techniques may be useful for studies with large sample cohorts and sequential analysis in a monitoring scenario that demand excellent reproducibility. Lastly, the choice of kit will be influenced by variations in the price and quantity of sample material available (A Kustanovich, 2019).

The recovery of exogenous spike-in fragments after ccfDNA extraction can be used to gauge the extraction's effectiveness. To guarantee comparable behavior in terms of denaturation, nonspecific binding, and precipitation, the spike-in control should have a fragment length comparable to ccfDNA (R Meddeb, 2019).

Quantification of ccfDNA and Patterns of Fragmentation:

The sensitivity of ctDNA analyses is significantly impacted by ccfDNA concentration. Furthermore, when using next-generation sequencing (NGS), precise ctDNA quantification is essential to producing an output that satisfies the desired coverage and depth. Fluorometry (e.g., Qubit, Invitrogen, Thermo Fisher Scientific) and PCR-based techniques (e.g., quantitative PCR (qPCR) and digital PCR (dPCR)) can be used to quantify the ccfDNA yield following extraction. While PCR-based assays focus on certain genes or genomic areas, fluorometry-based techniques measure all DNA globally. While PCR-based methods only amplify intact

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DNA, fluorometry-based approaches can overstate ccfDNA concentration since they also measure damaged DNA. Qubit can quantify ssDNA and dsDNA independently and provides quick findings. Although qPCR and dPCR have better sensitivity and specificity, measurement is more expensive and time-consuming (M Alcaide et al., 2020).

Examining the fragmentation patterns of ctDNA and noncancer ccfDNA can reveal whether the material is contaminated with HMW DNA. Microfluidic electrophoresis (e.g., TapeStation, Agilent Technologies) or PCR-based techniques that analyze two or more fragment sizes of a gene or genomic area can be used to evaluate the fragmentation patterns of the isolated ccfDNA. Longer fragments may be a sign of possible HMW DNA contamination because ctDNA is more fragmented than genomic DNA (H Wu et al., 2022).

Assessing Double-Stranded and Single-Stranded DNA:

After extraction, some of the ctDNA can be single-stranded. When dsDNA is required for sequencing library preparation in NGS, the inclusion of ssDNA may result in an overestimation of input DNA and inaccurate quantification by dPCR. To prevent bias in quantification, a fluorometric assay that precisely measures dsDNA can be employed for library construction. Before partitioning, the DNA can be fully denatured into ssDNA for more accurate and reliable dPCR quantification. The ratio of ssDNA to dsDNA in a sample can be determined by comparing the quantity of denatured and nondenatured DNA. This gives an idea of how much of the sample will be used for library preparation. This characteristic could differ amongst extraction kits and could be a crucial consideration when evaluating kits (R Meddeb, 2019).

Techniques for Identifying ctDNA in Cancer Patients:

Digital PCR-based techniques and next-generation sequencing (NGS)-based techniques are the two main approaches utilized for ctDNA detection. Both techniques can be used to examine one or more targets in tumor-informed or tumor-agnostic approaches. The analysis's sensitivity and specificity may be impacted by the selection of genetic and epigenetic markers. A tumor-informed single-target may be adequate in a postoperative MRD research, but only tumor-agnostic indicators can be used in a diagnostic setting, and assessing numerous targets can boost the test's sensitivity. Both approaches have benefits and drawbacks, and clinical application should be taken into consideration while selecting a strategy (F Mouliere et al., 2018).

PCR-Based Digital Methods:

Digital PCR is a quick, easy, and reasonably priced technique. Where amplification takes place, the target nucleic acid (NA) molecules are dispersed at random into many partitions with predetermined nanoliter or picoliter volumes. Digital PCR platforms come in two primary varieties: Target NAs are divided into small chambers in chamber-based digital PCR (cdPCR) and small droplets in droplet-based digital PCR (ddPCR)

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(J He et al., 2023).

Regular thermocycling conditions can amplify 0, 1, or more target NAs in each compartment. The presence or absence of the targeted NA, which is connected to a fluorescent dye in each compartment, can then be used to determine if the outcome is positive or negative. The results are based on end-point measurements because the amplification occurs inside the compartments. The Poisson distribution and partitioning statistics are the foundation of dPCR quantification, which is made possible by the large number of regions of opportunity (number of partitions) and the low likelihood of success (target NAs per partition) (D Kotani et al., 2023).

Clinical Application of ctDNA Detection:

When working with ctDNA, dPCR and NGS are frequently utilized both independently and in combination. Both methods have benefits and drawbacks. dPCR is faster, less costly, and requires less thorough data analysis than NGS. It is also quicker to put into practice. The restricted multiplexing capacity of dPCR in comparison to NGS and the requirement to know the NA target beforehand are drawbacks. NGS makes it possible to simultaneously examine several genomic regions and identify de novo mutations that may develop in subclones during therapy and, for example, result in treatment resistance. However, it is more costly than dPCR and necessitates sophisticated bioinformatics pipelines and tools to handle the massive volume of data (EA Klein et al., 2021).

Recommendations:

- To guarantee accurate quantification, issues such HMW DNA contamination are taken into consideration because the studies must be conducted under strict quality control. Comorbidities and lifestyle factors are considered to have an impact on ctDNA detection, although the literature is not conclusive. When assessing and reporting findings, these should be considered.
- Instead of reporting outcomes as positive or negative, they could be described as detected or undetected or informative or noninformative. The components will vary depending on the goal and technique of analysis. Quantitative results with CI are preferred if ctDNA data are used to track the course of cancer and customize treatment for each patient, even though a detected/undetected result could be adequate in an early detection or MRD setting or when screening for resistance mutations.
- A CI for the quantification should be provided together with the VAF and the quantity of ctDNA molecules/mL. Reporting the amount of ccfDNA/mL in the sample along with the determined maximum level of sensitivity for the assay may be pertinent for undetectable quantities of ctDNA.
- Clinicians should be made aware of putative germline variations and those that are probably not tumor-related (such as CHIP).

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Conclusion:

The wide use of ctDNA as a biomarker requires standardization of the preanalytical and analytical processes as well as result reports. To draw accurate inferences about quantitative changes in ctDNA levels in particular individuals, results must be similar over time. In order to avoid requiring individual laboratories to confirm all clinical applications of studies and specify cut-offs and actionable levels of ctDNA, results from various laboratories should be comparable. For ctDNA studies, dPCR and NGS are both extremely sensitive techniques. They are complementing techniques that can be applied at various stages of a patient's diagnosis and course of treatment. When choosing the best approach, factors including the required number of targets, sensitivity level, sample-to-result time, and cost should be considered.

The laboratory and the clinic should work together to translate ctDNA results from research into a therapeutically useful tool. To guarantee timely delivery of high-quality analyses, the laboratory must be informed of the clinical context and required time period. In order for physicians to apply the results in a clinical setting and optimize the benefit for patients, the results should be given with the pertinent information. The clinical utility of ctDNA analyses is becoming more and more clear, whether they are used alone or in combination with other assays. Selecting a ctDNA test to customize patient care may become commonplace in the near future and presents significant opportunities for personalized medicine.

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