LIQUID BIOPSIES-A MULTIMODAL CANCER DIAGNOSTIC TOOL

Abstract

Considering the challenges encountered in diagnostic methods related to conventional biopsies, a shift from traditional biopsies has been made possible through techniques such as liquid biopsy, empowering onco-pathologists with molecular profiling of tumour at all stage of its development. Liquid Biopsy targets comprises large or small molecules found in body fluids that lack cells or subcellular structures including proteins, nucleic acids, lipids, carbohydrates, small metabolites, and metal ions and targets with cellular or subcellular structures, such as single or clustered circulating tumor cells (CTCs), circulating cancer-related fibroblasts (CAF), immune cells, tumor-educated platelets (TEP), extracellular vesicles (EVs), and circulating mitochondria. These analytes can be assessed to gather information about both the overall tumor burden and the underlying biology, particularly as cancer cells undergo immunoediting and encounter evolutionary bottlenecks induced by therapeutic interventions. Pre-analytical variables may hamper the result of the analytes as the molecular characteristics of clinical samples can undergo significant alterations throughout the initial processing steps, encompassing everything from gathering the specimen to extracting the desired molecules like nucleic acids. Real time PCR, Digital PCR and Next Generation Sequencing are the tools by which these analytes can be analysed. Liquid biopsy testing has a highly attractive therapeutic use, but it is still confined to primary tumor identification, treatment response assessment, therapy monitoring, minimal residual disease detection, tumor heterogeneity characterization, and formation of resistances to targeted therapies.

Keywords: Liquid Biopsies, Cancer Diagnostic Tool, ctDNA and cfDNA, CTCs, Extracellular Vesicles.

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I. INTRODUCTION

Traditional tissue biopsies, Image guided or not, are considered as a gold standard in cancer diagnostics. They not only facilitate histological diagnosis but also analyse the molecular profiling of the tumour thereby improving the clinical outcome. It has been shown that molecular profiling of tumors taken from specific individuals can improve the choice of individualized cancer treatment treatments, forecast patient responses, detect drug resistance, and track tumor recurrence.(1-2)

However, the technique is invasive, and sometimes it is not easy to obtain it due to its anatomical location or the characteristics of the tumour. Furthermore, the tumours grow, mutate, and become heterogeneous resulting in inconsistencies in the test report(3). These invasive techniques for procuring tissue samples are also linked to a variety of other limitations inherent to the procedure. These include challenges in obtaining tumor samples that meet standards for both quantity and quality, as well as issues such as low sensitivity, high costs associated with sample isolation, and the need for clinical validation.

Furthermore, the process of obtaining biopsy samples through invasive methods throughout the course of treatment, for the purpose of monitoring tumor response and relapse, presents a significant hurdle in comprehensive tumor profiling. This challenge is particularly pronounced in cases of metastasis, where tumors have disseminated and continuously undergo spatial and temporal evolution in response to treatment over time. Consequently, the complex nature of tumor behavior may necessitate multiple biopsies to gain a comprehensive understanding, as it proves difficult to capture a holistic representation of the tumor (4).

Considering the challenges encountered in diagnostic methods related to conventional biopsies, the examination of different biological fluids for components originating from tumors has gained preference over the past decade. This shift from traditional biopsies has been made possible through techniques such as liquid biopsy, empowering onco-pathologists with molecular profiling of tumour at all stage of its development.

The term "Liquid Biopsy" was coined in 2010 by Pantel K and Catherine Alix-Panabières to describe blood draws used for assessing circulating tumor cells (CTCs). Over time, the term has expanded to encompass circulating plasma tumor DNA (5). Biologically, liquid biopsy targets can be categorized into two groups. The first category comprises large or small molecules found in body fluids that lack cells or subcellular structures. These molecules include proteins, nucleic acids, lipids, carbohydrates, small metabolites, and metal ions. The second category includes targets with cellular or subcellular structures, such as single or clustered circulating tumor cells (CTCs), circulating cancer-related fibroblasts (CAF), immune cells, tumor-educated platelets (TEP) (6), extracellular vesicles (EVs), and circulating mitochondria (7,8). These sources can be examined to gain insights into both the overall tumor load and the fundamental biology, especially as cancer cells undergo immunoediting and encounter evolutionary bottlenecks due to therapeutic interventions (9).

The primary benefit of liquid biopsy analysis lies in its minimal invasiveness and the potential for serial repetition, enabling real-time extraction of tumor-related information. Furthermore, the discovery of predictive biomarkers in peripheral blood, capable of monitoring therapy response in real time, presents a significant opportunity for innovative strategies in the therapeutic care of cancer patients.(3)

Any technique employed for the detection or quantification of disease-related biomarkers in human bodily fluids is referred to as a "liquid biopsy." While other bodily fluids like mucosa, pleural effusions, urine, and cerebrospinal fluid (CSF) are also examined, blood sampling is the primary approach in liquid biopsies (10). Liquid biopsy analytes encompass circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), the portion of cell-free DNA (cfDNA) derived from tumors, extracellular vesicles, tumor-educated platelets, proteins, and metabolites. These analytes can be assessed to gather information about both the overall tumor burden and the underlying biology, particularly as cancer cells undergo immunoediting and encounter evolutionary bottlenecks induced by therapeutic interventions (9).

II. CIRCULATING TUMOR CELLS (CTCs)

CTCs constitute a diverse group of cancer cells that detach from either the primary tumor or metastatic lesions at distant sites in patients. These cells initially stem from the primary tumors within the tissue, are subsequently released, and travel through the circulatory system. They hold a crucial role in the development of metastatic or secondary tumors in various distant parts of the body (11). It's worth noting that they exhibit a relatively short half-life, typically ranging from about 1 to 2.4 hours.

These cells are very heterogenous, and not only varies in numbers from patient to patient but also vary within the same patient from time to time. In numerical terms, their presence in the bloodstream is relatively low, with approximately one CTC detected per million leukocytes (12). Regarding their morphology, research has indicated that the shape of CTCs varies based on the stage and/or type of tumor.9 Furthermore, CTCs have been observed to aggregate by adhering to cells such as fibroblasts, platelets, and others. These aggregates have been documented to disseminate to more distant locations within the body compared to individual CTCs. This clustering of cells provides them protection against oxidative stress and the immune response in their surroundings (13,14,15).

III. CIRCULATING TUMOR DNA (ctDNA) AND CIRCULATING CELL FREE DNA (cfDNA)

Usually, when cells undergo apoptosis or necrosis, they release nuclear and mitochondrial DNA into the bloodstream as part of the cellular breakdown process. This released DNA is subsequently eliminated from the circulation by phagocytes. Hence the level of these circulating DNA fragments is very low in a healthy individual (16,17).

Circulating tumour cells may release ctDNA in the circulation. Several hypotheses have been put forth to elucidate why viable cancer cells might intentionally release DNA into the bloodstream. One such possibility is that cancer cells release oncogenic DNA with the intention of influencing the transformation of susceptible cells at distant sites (16). It has also been found that effective clearance of these fragments by Phagocytosis may not happen in certain instances like severe inflammation, excessive exercise or molecular changes within a tunoral mass resulting in accumulation of cell debris and DNA fragments which ultimately is released into the circulation (17).

Nevertheless, CTCs do not exclusively contribute to the presence of ctDNA. In a single human cell, there exists approximately 6 pg of DNA, and there's an average of 17 ng of DNA per milliliter of plasma in individuals with advanced-stage cancers (18, 19, 20). Therefore, if CTCs were the primary origin of ctDNA, it would necessitate the presence of more than 2,000 cells per milliliter of plasma. However, in practice, the average count of CTCs is less than 10 per 7.5 milliliters of blood (21). Additionally, physiological conditions such as inflammation or physical exercise are known to elevate cfDNA levels, which may not always indicate an underlying malignancy (22, 23).

It's important to emphasize that ctDNA constitutes only a fraction of the total circulating cell-free DNA (cfDNA), which typically ranges from 10 to 100 ng/ml in normal plasma levels (24). Both the quantity and integrity of circulating cfDNA can serve as differentiating factors between cancer patients and those who are healthy. Generally, cancer patients exhibit higher levels of cfDNA compared to healthy individuals, and these levels tend to increase with the stage and presence of metastasis (25, 26). Recent research findings have indicated that the length of ctDNA fragments in cancer patients falls within the range of 20-50 base pairs, which is notably shorter than cfDNA (27).

IV. EXTRACELLULAR VESICLES

Membrane-bound particles known as extracellular vesicles (EVs) are produced by all cell types, both healthily and diseasedly. Proteases, ADP, thrombin, inflammatory cytokines, growth factors, biomechanical shear and stress inducers, and apoptotic signals are among the stimuli that cause them to be formed (28).

Based on their composition and secretory mechanisms, these entities have been categorized into microvesicles and exosomes. Extracellular vesicles (EVs) are small, membrane-bound vesicles with a saucer-like shape, typically measuring around 30–100 nm in size. They can be found in various bodily fluids, including plasma, urine, cerebrospinal fluid (CSF), saliva, and others (29). The release of these vesicles from cells is believed to be influenced by the mitogen-activated protein kinase pathway, which is often up-regulated in many tumor cells (30). These vesicles exhibit distinct markers such as CD9, CD63, CD81, ALIX, and heat shock protein 70 (HSP70), which aid in their isolation and enrichment (31).

Extracellular Vesicles carry diverse biomolecules like DNA, RNA, protein, etc., and play crucial role in intercellular communication. They attains high levels in body fluids as they are secreted profusely by the tumour cells (31). Analysis of EVs helps in analyzing tumor monitoring, prognosis, and therapeutic response (32,33). Most cells release exosomes, which are tiny extracellular vesicles that range in size from 40 to 160 nm and are crucial for intercellular communication (34).

Exosomes are derived from the endosome system and are responsible for transferring crucial information between cells by carrying a range of substances such as proteins, lipids, nucleic acids (DNA, microRNA, and mRNA), and other pertinent information (35). Studies have shown that because exosomes are present in nearly all bodily fluids, they may represent a potential biomarker in liquidbiopsy. However, the absence of improved standard techniques for component analysis and separation has restricted the clinical applicability..

1. **miRNA:** MicroRNAs (miRNAs) are a class of non-coding RNAs approximately 18–25 nucleotides in length, serving regulatory roles. Notably, miRNAs govern the expression of diverse oncogenes and tumor-suppressor genes, exerting a pivotal influence on the pathological progression of tumor development (36,37).

   As a result, changed levels of miRNA that circulate in peripheral blood are linked to the emergence of cancer. Extensive research has shown that miRNA in exosomes may be a useful non-invasive biomarker for predicting cancer outcome and risk stratification (38,39, 40).

   When it comes to miRNA analysis, quantitative real-time PCR (qRT-PCR) is the gold standard method. Its clinical applicability is, however, limited by many drawbacks, including non-absolute measurement, false positives, and costly equipment.

2. **TEPs:** Platelets have a complex function and use a number of methods to encourage metastasis. They attract stromal cells, shield CTCs from shear pressures and immune surveillance, and promote tumor angiogenesis and vascular remodeling. Conversely, tumors teach platelets and cause them to activate, aggregate, and discharge chemicals produced from platelets into the bloodstream. They also impact megakaryopoiesis in the bone marrow, which in turn promotes thrombocytosis.

   In the bidirectional interactions between tumors and platelets, platelets exhibit both systemic and localized responses to cancer. They continuously uptake and enhance their content with free proteins, nucleic acids, vesicles, and particles (41,42), consequently causing changes in their RNA and proteomics expression patterns . This phenomenon is referred to as "tumor-educated platelets" (TEPs) (43).

   Tumor-educated platelets are defined as functional platelets with a distinct tumor-driven phenotype due to the transfer of tumor-related molecules from cancer cells to platelets (44).. Nonetheless, the process by which platelets undergo education and attain unique RNA and protein profiles remains incompletely understood. The majority of conducted research has concentrated on the RNA content of tumor-educated platelets (TEPs), revealing that distinctive spliced TEP-RNA signatures can furnish specific insights into the existence of cancer cells and contribute to tumor progression (45,46).

V. SAMPLING PROCEDURE FOR LIQUID BIOPSIES

An experienced phlebotomist should take the sample in order to isolate biomarkers from the blood and prevent hemolysis. When isolating cfDNA, plasma is preferred over serum. To collect samples, standard K2- or K3-EDTA tubes can be utilized. Plasma should be collected as soon as possible following blood withdrawal, and blood should not be kept in EDTA tubes at room temperature for longer than three hours. Whole blood kept at 4°C is useless since it doesn't stop leukocyte lysis.

Use particular preservation tubes with unique fixatives that can stabilize blood and cfDNA for a few days if processing the material within three hours of collection is not feasible.The amount of blood needed to produce a significant amount of ctDNA is still unknown, however many diagnostic kits specify the minimal amount of plasma needed for analysis.

Two steps centrifugation must be done to obtain plasma so that no cell residues is left. The first step should be a low speed centrifugation with a speed of 1200-1600 g to avoid leukocyte lysis. the supernatant is separated and again subjected to a second step 0f high-speed centrifugation with a speed of≥3000 g to remove all contaminants. Centrifugations must be carried out without any pause in between. The use of a refrigerated centrifuge (4°C) is also recommended.

The produced plasma can be kept for a brief time (about one month) at −20°C. To ensure cfDNA stability over extended periods, plasma should be stored at −80°C to prevent freezing and thawing cycles, which can consistently reduce the amount of total cfDNA (47, 48).

For other analytes like mucosa, pleural effusions, urine, and cerebrospinal fluid (CSF), the selection of the appropriate body fluid for a liquid biopsy must be a meticulously considered decision, particularly in cases where the technique is invasive like CSF and where certain fluids might not accurately reflect the tumor's origin. It is always imperative to capitalize on the primary advantage of liquid biopsy, which lies in its ability to procure samples with minimal invasiveness.

It is better to centrifuge the CSF sample in a refrigerated centrifuge at a speed of 400 g for 5 min at 4°C or 2000g for 5 min at 4°C followed by 10000g for 5 min at 4°C. The supernatant can then be divided into 1mL aliquots and stored at -80°C (49).

Pre-analytical variables may hamper the result of the analytes as the molecular characteristics of clinical samples can undergo significant alterations throughout the initial processing steps, encompassing everything from gathering the specimen to extracting the desired molecules like nucleic acids.

This also includes transporting, storing, and archiving the sample, as well as extracting the molecules present in it. These modifications can lead to changes in the intended analytical outcomes. Without a well-defined and carefully followed process, there is a notable risk that the diagnostic tests might not accurately reflect the original analyte composition within the patient's body. Instead, the tests might end up measuring altered levels resulting from the processing steps. Hence for all types of liquid biopsy sample, standardized and validated steps to be performed to achieve reliable diagnostic outcomes (50).

VI. TECHNOLOGIES FOR THE ANALYSIS OF LIQUID BIOBSY

Five tests have been approved by the FDA since the discovery of cfDNA. These assays include the identification of point mutations in genes linked to cancer, including EGFR, PIK3CA, and KRAS. They also include the assessment of ALK rearrangement, insertions and deletions, microsatellite instability, methylation patterns, and tumor mutation burden (TMB) ((51).Several methods can be applied to liquid biopsy samples, such as multigene NGS-panel, digital PCR, or real-time PCR. The outcomes typically exhibit strong specificity and a broad spectrum of sensitivity rates. The exact sensitivity levels depend on factors such as the nature of the sample, the clinical attributes of the patients, and the characteristics of the tumor.

1. **Real-Time PCR:** Currently, point mutations and/or small insertions and deletions in ctDNA are best analyzed using real-time PCR. This method is generally available in molecular diagnostic labs since it can guarantee sensitivity and a quick turnaround time (TAT) at a reasonable price.

   Real-time PCR makes use of a specialized probe that can be customized to improve diagnostic sensitivity, as demonstrated by the amplification refractory mutation system (ARMS/Scorpion) technology. This technology enhances sensitivity by simultaneously amplifying mutated alleles of the target gene or genes of interest alongside an internal control gene. Furthermore, a unique combination of control oligonucleotides enables the evaluation of both DNA quality and quantity. By employing this specific technology, it becomes possible to achieve an exceptionally low limit of detection (LOD), reaching as low as 0.5%. Consequently, this approach is highly suitable for detecting minor proportions of mutated alleles within significant quantities of wild-type genomic DNA, particularly in the context of ctDNA (52).

2. **Digital PCR:** Digital PCR (dPCR) represents a technological advancement building upon the conventional PCR. This innovative approach aims to convert the exponential, analog nature of PCR into a linear, digital (or binary) signal (53).

   There are three types of dPCR platforms:

   - **Droplet dPCR (ddPCR)**, where an oil-water emulsion's about 20,000 uniform droplets are used to represent partitions. Using the Poisson distribution, individual DNA molecules are dispersed across the bioreactors (droplets or wells) (54).

   - **Solid Digital PCR (sdPCR)** where the bioreactors are portrayed through approximately 20,000 to 12,000 partitions that are positioned on a solid support (chip). This approach eliminates the need for an emulsion procedure and mitigates the risk of droplet breakage, which could otherwise result in a reduction in analysis performance (55). DNA molecules are spread similar to the droplet dPCR system.

   - **Beam, Emulsion, Amplification, Magnetics (BEAMing) dPCR**. Here, an initial standard PCR amplification step is necessary prior to conducting the analysis (56). The enhanced products are then distributed into many homogeneous droplets that are created within an oil-water emulsion. Additionally, magnetic microspheres that bind the PCR products are injected. The beads are then separated using magnetic separation or centrifugation. Eventually, optical scanning or flow cytometry can be used to quantify the DNA linked to the microspheres, resulting in a limit of detection (LOD) of 0.01%.eighteen

Fluorescence detection is used to analyze the analytes after the PCR amplification stages are complete. By dividing the amplification response, both qualitative and quantitative information about even tiny numbers of mutant genes in a background of wild-type alleles can be retrieved. dPCR has higher sensitivity compared to real-time PCR, with an accuracy and reproducibility of 0.1%-0.01% at the sensitivity limit (54).

The detection limit of ctDNA mutations has been lowered to 0.01%–0.02% thanks to ddPCR and BEAMing, whereas the sensitivity (82%–87%) and specificity (97%) for EGFR-sensitizing mutations in lung cancer are comparable (57,58). Although it exhibits extremely sensitive and focused performances, its workflow is costly and difficult to use in standard clinical settings (59).

A primary constraint inherent in all digital PCR methods, when contrasted with sequencing-based techniques, is their limitation in detecting solely established mutations. This limitation hinders the ability to identify novel alterations (60).

Notwithstanding these drawbacks, dPCR is a reliable confirmatory technique whose utility as a stand-in biomarker for treatment response is strengthened by its capacity to quantify the altered alleles and, consequently, to track patients over time (60).

When encountering challenging result interpretations, provided that pre-analytical and analytical concerns have been ruled out, it is advisable to assess the clinical parameters. Conditions such as a low disease burden or the progression of disease in the brain or bones typically exhibit minimal ctDNA shedding in the body fluids. This can potentially lead to misinterpretation of results (61).

VII. NEXT-GENERATION SEQUENCING

Multiple genes along with multiple known and unknown alterations such as single-nucleotide variant (SNV), insertions/deletions, rearrangements can be investigated simultaneously with Next Generation Sequencing.

Newer and more sensitive NGS applications based on targeted sequencing allow a sensitivity <1% (0.1%-0.01%) for ctDNA analysis. The tagged-amplicon (TAm-seq) and its more sophisticated variant, the eTAmSeqTM; the safe-sequencing system (Safe-SeqS); and the CAncer Personalized Profiling (CAPP-seq), or AmpliSeq, are examples of this type of targeted sequencing. Certain panels have the capability to examine circulating DNA and RNA, as well as complex modifications such MET amplification (34, 35, 36).

The concordance between NGS analysis of tumour metastasis and ctDNA has been found to be 97%'as compared to low concordance between primary tissue and ctDNA which is primarily due to tumour heterogeneity or clonal hematopoiesis which increases with age (37,38).

Yi Chen and colleagues investigated the new functions of liquid biopsies based on NGS in non-small cell lung cancer. Researchers came to the conclusion that NGS-based ctDNA assays might be used to detect actionable genetic alterations, follow the spatiotemporal progression of lung cancer, and dynamically assess response and resistance to targeted treatments. Nevertheless, complexities associated with discerning clinically significant driver genomic changes, establishing meaningful cutoff frequencies for clinical relevance, difficulties in identifying predominant resistance mechanisms, determining the appropriate course of action upon obtaining positive ctDNA screening outcomes in early-stage non-small cell lung cancer (NSCLC), and evaluating the cost-effectiveness of managing tumor evolution are some of the challenges faced (62).

VIII. REPORT OF LB RESULTS

The integral part of all the diagnostic procedures is the reliable reporting based on which the treatment modalities are decided. Liquid biopsy reports must include the following information:

- unique identification of the patient
- identification of the unit/physician which prescribed the analysis
- material used for the analysis (type and volume) and date of collection
- methods of sample storage
- sample acceptance date and date of reporting
- methods used for analysis
- investigated variants (for targeted assays)
- genes covered (for the untargeted assay)
- test results
- sensitivity, specificity and LOD of the assay
- data interpretation regarding druggability, actionability and resistance profiles

The report needs to be filled out using a predefined template with all the above mentioned requisites, with a date and the signature of the laboratory Specialist. Recognizing the test's significance for treatment planning, the reporting timeframe should not surpass five days from the time of the request. Due to the general diagnostic sensitivity of liquid biopsy (approximately 87%), instances of negative results for mutations should not be categorized as 'wild-type', as the potential for false negatives always exists. Hence, when faced with a negative outcome and when technically possible, it is advisable to consider utilizing a tissue biopsy or potentially conducting a second liquid biopsy procedure.

IX. LIMITATIONS

Liquid biopsies hold great promise as a strategy for advancing biomarker research. However, before these biomarkers can find their way into clinical practice, several technical, biological, and clinical challenges must be navigated. Challenges such as the absence of universally standardized assays and the substantial cost associated with liquid biopsies impede the seamless integration of these biomarkers into clinical workflows. Furthermore, the utility of liquid biopsy techniques has often been explored in studies with limited sample sizes, underscoring the necessity for extensive, high-quality research on a larger scale. This rigorous approach is crucial to corroborate earlier findings and establish the foundation for the routine clinical utilization of liquid biomarkers (63).

Special emphasis should be given on precise and validated standard procedures for sample collection and targeted analytes during the pre analytical phase. Specimen collection devices with stabilizing agents for the analytes and kits for isolating nucleic acids should be well checked. duriDiagnostic companies and diagnostic laboratories are increasingly focused on specified and verified generic pre-analytical workflows for different specimen types and analytical targets. Links to state-of-the-art, well-designed, specified, and verified analytical test technologies including related instrument platforms (such as NGS dPCR with verified software and integrated post-analytical steps such as bioinformatics for data analysis and interpretation should be made available. All are increasingly becoming the building blocks and basis for the development of new, safe, and reliable analytical tests.

Liquid biopsy testing has a highly attractive therapeutic use, but it is still confined to primary tumor identification, treatment response assessment, therapy monitoring, minimal residual disease detection, tumor heterogeneity characterization, and formation of resistances to targeted therapies (64).

In a broader context, the widespread integration of liquid biopsy into clinical practice demands a comprehensive grasp of its complete capabilities and constraints. This comprehensive understanding can only be attained through the meticulous analysis of extensive patient groups spanning various cancer categories and encompassing multiple medical facilities (65). Despite this requisite, the elevated anticipations surrounding liquid biopsy as an innovative instrument for both cancer detection and surveillance are anticipated to galvanize intensified exploration in this domain in the forthcoming years. The overarching aim is to tackle the persisting uncertainties and ultimately determine whether liquid biopsy will stand as a pivotal advancement in the care of individuals afflicted by cancer.

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