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Liquid Biopsy in Cancer Monitoring: Beyond Circulating Tumor DNA

Hajra Ahmad[1*] | Irshad Ali[1] | Mubashra Khadim[1]

[1]Department of Medical Laboratory Sciences, UHS, Lahore, Pakistan

***Correspondence:** Hajra Ahmad (hajraahmadmls@gmail.com)

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ABSTRACT

Background: Liquid biopsy has become a potent, least invasive way of detecting, monitoring, and treating cancer by studying cancer-causing biomarkers in body fluids. The circulating tumor DNA (ctDNA) has long been the foundation of the liquid biopsy as it has the capability of measuring tumor-specific genetic changes. New developments have made it possible to identify and characterize a wide range of circulating analytes, such as ctDNA, extracellular vesicles, circulating RNA, tumor-educated platelets, and circulating proteins and metabolites. These biomarkers are associated with tumor viability, transcriptional activity, metastatic potential, and metabolic reprogramming. Innovations in next-generation sequencing, microfluidics, proteomics, and single-cell analysis have led to the detection platforms of many multi-analytes being immensely sensitive and reliable. Integrative liquid biopsy platforms can help to assess the evolution and therapeutic resistance of tumors in a holistic manner through the integration of genomic, transcriptomic, and proteomic and cellular data. Moreover, data interpretation has also been improved with the implementation of artificial intelligence and machine learning, resulting in reliable classification of the disease, risks stratification, and predict the response to treatment. Although significant progress has been made, complications such as, variability in analysis, absence of standardized protocols, high cost of operation, and biological heterogeneity, restrict the extensive clinical implementation. Technological advancement and interdisciplinary cooperation will continue to intensify the role of liquid biopsy in achieving the possibility of early detection, real-time monitoring, and optimal cancer management.

Keywords: Liquid Biopsy; Neoplasms; Circulating Tumor DNA; Extracellular Vesicles; RNA, Circulating; Platelets; Proteomics; High-Throughput Nucleotide Sequencing

Cancer is currently among the major morbid and mortal causes in the world, and causes millions of deaths annually despite the considerable improvements in diagnostic and treatment modalities [1]. The timely monitoring of the disease and proper treatment response, as well as early detection, are key factors that define patient survival and quality of life [2]. Conventionally, the diagnosis of tumors has been based on biopsy of tissues, radiographic studies and physical examination [3]. Despite the fact that tissue biopsy is the gold standard in cancer diagnosis, it is not only invasive but also expensive and usually not practicable when repeated cancer monitoring is required [4]. In addition, the spatial and temporal heterogeneity of tumors makes it impossible to use a single-site biopsy to comprehensively describe the dynamic molecular features of malignancies [5]. Liquid biopsy, in turn, is a new promising minimally invasive technique of real-time cancer monitoring, discovered in recent years [6]. Liquid biopsy can be used to detect tumor-derived components in the body fluids, where a single sample could be analyzed repeatedly, and disease progression can be assessed longitudinally. Circulating tumor DNA (ctDNA) is one of the most studied circulating biomarkers that have been widely utilized in the field of early detection, monitoring of minimal residual disease, therapeutic targeting, and resistance mutations [7]. The clinical utility of the method has been already marked by several ctDNA-based tests being introduced into clinical practice [8].

In spite of these developments, the use of ctDNA on its own is subjected to significant limitations. ctDNA detection is sensitive depending on the types of tumors, the stage of the disease and the tumor burden. In early stages of cancer, tumors have low shedding rates and ctDNA levels might not be satisfactory to detect cancers and tumors. Moreover, biological factors including clearance, fragmentation, and dilution of non-tumor cell-free DNA affect the reliable interpretation [9]. These limitations highlight the importance of complementary biomarkers that could improve the strength and dependability of the cancer monitoring by liquid biopsy. There is an emerging literature that various tumor-derived products such as circulating tumor cells, extracellular vesicles, circulating RNA, tumor-guided platelets and tumor-associated proteins and metabolites offer useful and distinct biological data [10]. All the mentioned biomarkers represent varying components of tumor biology including

cellular viability, metastatic potential, gene expression dynamics, and metabolic activity. Combining these various signals could provide a more detailed and sensitive evaluation of the tumor dynamics than that provided by ctDNA alone. This scientific communication aims to cover the growing field of liquid biopsy beyond circulating tumor DNA, with a specific focus on novel biomarkers and their applicability in cancer monitoring. Through detailed analysis of existing data and suggestion of a unified multi-analyte model, the article aims to outline key future perspectives of enhancing personalized cancer treatment by incorporating superior liquid biopsy protocols.

Current Status of Liquid Biopsy, The Circulating Tumor DNA Era: Analysis of ctDNA has played a major role in the clinical implementation of liquid biopsy, as these DNA fragments are discharged into the blood through apoptosis, necrosis, and active release by tumor cells [11]. These fragments bear tumor-specific changes at genetic and epigenetic level, such as point mutations, copy number, chromosome rearrangement, and methylation patterns, leading to disease variability across different types of cancer [12]. Consequently, ctDNA gives us a non-invasive glimpse into the molecular structure of cancer. Digital polymerase chain reaction (dPCR), droplet digital PCR (ddPCR) and next-generation sequencing (NGS) are technological innovations that have greatly improved the sensitivity and specificity of ctDNA detection. On these platforms, rare tumor variants in a high background of normal cell-free DNA can be identified. ctDNA testing has gained utility in the precision oncology domain, especially in terms of molecular profiling and therapeutic decision-making. A number of commercial tests, such as Guardant360 and FoundationOne Liquid CDx, have been approved by the regulators and are now being used in clinical practice on a regular basis [13]. In the clinical context, ctDNA has been found to be useful in various cancer types. ctDNA profiling can also be used to identify alterations in advanced malignancies to enable clinicians to create targeted treatments. In the early disease detection, ctDNA has demonstrated potential in the detection of minimal residual disease (MRD) after surgery or chemotherapy. Elevated ctDNA concentrations commonly begin before the radiological signs of a relapse and thus allow prompt therapeutic response [14].

In addition, treatment response and prognosis have been linked to dynamic changes in the ctDNA burden during treatment. Notwithstanding these accomplishments, a number of critical constraints of the universal viability of ctDNA-based monitoring exist. The release of tumor-derived DNA in the circulation is extremely changeable and relies on the size of the tumor, its vascularization, position, and biological response. The low ctDNA shedding that occurs in cancers of central nervous system and some indolent tumors often leads to false-negative. Also, clonal hematopoiesis of indeterminate potential (CHIP) may cause confounding mutations which are not associated with malignancy resulting in misinterpretation of data [15]. ctDNA is also difficult to assess due to analytical challenges. Pre-analytical factors, such as blood collection, processing time, storage conditions, and DNA extraction methods, have a great impact on the test performance [16]. Additionally, cross-laboratory standardization is not very pronounced and clinical harmonization is not extensive. Accessibility to high-cost sequencing and the complicated bioinformatic needs are limited in a resource-constrained environments [17]. Taken together, although ctDNA is one of the pillars of contemporary liquid biopsy, its biological and technical limitations have underscored the importance of using complementary biomarkers. It is thus important to adopt a multi-dimensional approach to capture the full range of tumor heterogeneity in order to enhance reliability of non-invasive monitoring of cancer.

Emerging Liquid Biopsy Biomarkers Beyond Circulating Tumor DNA: To address the shortcomings of the ctDNA-based monitoring, more efforts have focused on alternative circulating biomarkers, which exhibit a wide range of phenotypes of tumor biology. These novel analytes complement each other in terms of the tumor viability, metastatic potential, transcriptional activity, and tumor-host interactions. The ability to integrate these biomarkers in liquid biopsy-based systems can significantly improve the diagnostic sensitivity and clinical utility.

Circulating Tumor Cells: The circulating tumor cells (CTCs) are viable malignant cells that dissociate with the primary or metastatic lesions and find their way into the bloodstream [18]. In contrast to ctDNA, which is a fragment of genetic material, CTCs can offer information on viable cancer cells in terms of both function and morphology [19]. They have been linked to the development of the disease, metastasis and poor prognosis in various types of cancer such as breast, prostate, colorectal, and lung cancers. Characterization of phenotypes, genomic profiling and measurement of epithelial mesenchymal transition and stemness indicators can be achieved with the help of CTC analysis [20]. Moreover, ex-vivo culture and drug sensitivity tests of CTCs provide the promising results of personalized therapy. Their very low abundance and heterogeneity in phenotype, however, is a significant technical problem to isolation and analysis, so their use in clinical practice is limited [21].

Extracellular Vesicles and Exosomes: Extracellular Vesicles (EVs) and especially exosomes are membrane-enclosed nanoparticles that are released by tumor cells into the bloodstream [22]. All these vesicles are loaded with various types of molecular cargo with DNA, RNA, proteins, lipids, and metabolites, each of which depicts the physiological state of the cell proliferation rate. Tumor exosomes have a significant role in cancer development and transit in immune regulation, angiogenesis, and metastatic niche [23]. The use of exosomal biomarkers is characterized by a high degree of stability in biological fluids because of its protection by lipid bilayers. Their stability makes them attractive candidates in the application of liquid biopsy [24]. The diagnostic and prognostic relevance of exosomal microRNAs, long non-coding RNAs, and proteins in different malignancies have been proved [25]. However, there are still no standardized isolation and characterization procedures, which limit their utility in clinical translation.

Circulating RNA Biomarkers: Another potential type of liquid biopsy biomarker is circulating RNA species, such as messenger RNA, microRNA, long non-coding RNA, and circular RNA [26]. The expression patterns of these molecules indicate the existence of active gene regulatory networks and expression in tumor cells. Aberrant RNA expression profiles have been linked to tumor progression, metastasis, as well as treatment resistance [27]. MicroRNAs have received particular attention because of their stability and disease-specific expression pattern. The RNA-based assays have demonstrated possibilities of early cancer diagnosis and treatment monitoring [28]. Nevertheless, the low abundance and degradation of RNA, and the inconsistency of methods limit the inter-study reproducibility.

Tumor-Educated Platelets: Tumor-educated platelets (TEPs) are recently explored, novel type of biomarker. Direct interactions of platelets with tumor cells and uptake of tumor-derived molecules leads to molecular reprogramming of platelets altering RNA and protein profiles [29]. The changes allow TEPs to be dynamic precursors of tumor activity and presence [30]. Recent studies have shown that RNA signatures in platelets are able to differentiate cancer patients and healthy individuals, determine the origin of tumors and track the extent of treatment [31]. TEP-based diagnostics have merits associated with abundance and availability; however, its biological complexity requires additional mechanistic and clinical support.

Circulating Proteins and Metabolites: Additional biological layers are tumor-associated proteins and metabolites that are observed in serum or plasma [32]. The clinical use of protein biomarkers like prostate-specific antigen, CA-125 and carcinoembryonic antigen has continued to prevail

in clinical practice [33]. The proteomics and metabolomics have identified a list of detectable biomolecules, allowing to detect tumor-related pathways [34]. One of the characteristics of cancer is metabolic reprogramming; circulating metabolites change according to the needs of the tumor energy and its environmental adaptation [35]. The combination of proteomic and metabolomic data and genomic information could help to improve the characterization of disease and monitor therapy. Table 1 provides a concise comparative overview of emerging liquid biopsy biomarkers beyond circulating tumor DNA, their biological characteristics, clinical applications, advantages, limitations, and supporting evidence.

Table 1: Emerging Liquid Biopsy Biomarkers Beyond Circulating Tumor DNA and Their Clinical Significance

Biomarker	Biological Source / Nature	Key Molecular Cargo / Features	Clinical Applications	Major Advantages	Key Limitations
ctDNA [7, 11–17]	Fragmented tumor-derived DNA released via apoptosis, necrosis, and active secretion	Genetic and epigenetic alterations (mutations, CNVs, rearrangements, methylation)	Early detection, MRD monitoring, treatment response, resistance mutation identification, molecular profiling	Non-invasive molecular snapshot of tumor genome; enables precision oncology decision-making	Low shedding in early stage and CNS tumors; CHIP interference; pre-analytical variability; high sequencing cost
CTCs [18–21]	Viable malignant cells detached from primary or metastatic tumors	Morphological and functional tumor cell characteristics; EMT and stemness markers	Prognostic stratification, metastasis evaluation, phenotypic/genomic profiling, drug sensitivity testing	Provides viable cellular information and functional assays; reflects metastatic potential	Extremely low abundance; phenotypic heterogeneity; technical isolation challenges
Extracellular Vesicles [22–25]	Membrane-bound vesicles secreted by tumor cells	DNA, RNA, proteins, lipids, metabolites reflecting parental cell physiology	Diagnosis, prognosis, immune modulation monitoring, metastatic niche formation analysis	High stability due to lipid bilayer protection; abundant cargo diversity	Lack of standardized isolation and characterization methods
Circulating RNA (miRNA, mRNA, lncRNA, circRNA) [26–28]	Tumor-derived RNA fragments circulating in plasma/serum or vesicles	Gene expression signatures reflecting transcriptional activity	Early detection, treatment monitoring, metastasis and resistance prediction	Disease-specific expression; microRNA stability in fluids	Low abundance; RNA degradation; methodological inconsistency
TEPs [29–31]	Platelets reprogrammed through tumor interaction and molecular uptake	Altered RNA and protein signatures indicating tumor presence and activity	Cancer detection, tumor localization, therapy monitoring	High abundance and accessibility; dynamic tumor interaction indicator	Biological complexity; limited mechanistic understanding; need for validation
Circulating Proteins [32–34]	Tumor-associated secreted or shed proteins in serum/plasma	Classical tumor markers (PSA, CA-125, CEA) and proteomic signatures	Screening, prognosis, therapy monitoring	Established clinical utility; measurable via routine assays	Limited specificity and sensitivity when used alone
Circulating Metabolites [34–35]	Tumor-induced metabolic products detectable in body fluids	Metabolic pathway signatures reflecting tumor metabolic reprogramming	Tumor characterization, therapy response monitoring, metabolic pathway analysis	Captures tumor metabolic phenotype; complements genomic data	Biological variability; need for integration with multi-omics data
Multi-Analyte Integrative Platforms [36–48]	Combined analysis of ctDNA, CTCs, EVs, RNA, TEPs, proteins, metabolites	Multi-omics profiling integrating genomic, transcriptomic, proteomic, and metabolic signals	Early detection, MRD monitoring, therapy selection, resistance tracking, precision oncology	Holistic tumor characterization; improved sensitivity and predictive accuracy	Data integration complexity; lack of standardization; high cost; analytical reproducibility issues

Abbreviations: *lncRNA — long non-coding RNA; circRNA — circular RNA; TEPs — tumor-educated platelets; EMT — epithelial–mesenchymal transition; CNVs — copy number variations; MRD — minimal residual disease; CNS — central nervous system; CHIP — clonal hematopoiesis of indeterminate potential; PSA — prostate-specific antigen; CA-125 — cancer antigen 125; CEA — carcinoembryonic antigen; NGS — next-generation sequencing; ddPCR — droplet digital polymerase chain reaction.*

Integrative Multi-Analyte Approaches and Clinical Applications: Although the individual liquid biopsy biomarkers can give useful information about the biology of the tumor, dependence on a single biomarker can frequently restrict the sensitivity of the diagnosis and clinical utility of the technique [36]. The heterogeneity of tumors, dynamic clonal evolution and shedding patterns of biomarkers are variable and require a more detailed analytical framework [37]. Therefore, integrative multi-analyte liquid biopsy, has become an attractive solution in order to improve cancer detection, cancer monitoring, and therapeutic decision-making. Multi-analyte systems integrate data based on ctDNA, circulating tumor cells, extracellular vesicles, circulating RNA, tumor educated platelets and circulating proteins or metabolites [38]. This is an integrative methodology that allows the simultaneous determination in genetic alterations, transcriptional activity, cellular phenotypes, and metabolic signatures with a more holistic depiction of tumor status and disease progression with comprehensive profiling [39,40].

Recent technological improvements have generated sensitive platforms that can conduct parallel biomarkers examination. High throughput sequencing, digital PCR, mass spectrometry and microfluidic technologies enable the accurate determination of a variety of analytes in small sample volumes. In addition, single cell sequencing of CTCs and multi-omics of extracellular vesicles have increased the chances of real-time tumor evaluation and resistance to therapies [41]. Machine learning algorithms and artificial intelligence are becoming a more significant part of complex multi-dimensional data interpretation [42]. By combining genomic, transcriptomic, proteomic, and clinical data, these computational tools help in identifying the patterns, risk stratification, and predicting the models [43]. Classifiers based on machine learning have shown better accuracy in cancer diagnosis, localization and prediction of responses to treatment than single-marker-based methods [44]. Clinically, the integrative liquid biopsy platforms have demonstrated a lot of potential in various phases of cancer management. Multi-analyte assays are more sensitive in the early detection of small residual disease and occult malignancies as well as timely detection of therapeutic resistance and disease relapse [45,46]. During precision oncology, complex molecular profiling is applied as the choice of treatment for adaptive therapy [47].

A number of commercial and experimental multi-analyte tests have shown encouraging results in clinical trials, especially on colorectal, lung, breast, and hematological cancer. Such tests show that it is possible to translate integrative liquid biopsy technologies into a real clinical setting [48]. Nevertheless, massive validation research and regulatory standardization are the key to the mainstream adoption. Although there has been a lot of progress, issues of data integration, assays reproducibility, cost-effectiveness and clinical interpretation still remain. There exists inter-laboratory variability, lack of reference standards and heterogeneous pipelines of analysis which are hindering comparability across studies. To overcome these barriers, it is necessary to have synergy between clinicians, researchers, regulators and industry players. All in all, integrative multi-analyte liquid biopsy methods are the paradigm shift in cancer diagnostics and monitoring. These platforms have the potential to transform the face of early tumor detection, personalized treatment, and real-time monitoring of the disease by providing the multidimensional aspects of tumor biology.

Challenges, Limitations, and Future Perspectives: Although the liquid biopsy technologies have been rapidly developed and their clinical relevance has increased, there are a number of scientific, technical, and translation problems that still restrict their large-scale acceptance. The strategies to overcome these constraints are critical to the successful introduction of the highest-level liquid biopsy systems into the standard oncology practice. Analytical sensitivity and specificity are among the major challenges [49]. Numerous circulating biomarkers, especially circulating tumor cells and tumor-produced extracellular vesicles are found in very low concentrations, especially in early-stage disease. Poor detection leads to the probability of false-negative outcomes [50]. On the other hand, non-tumor-derived nucleic acid and proteins give background noise and can also show up as false-positive result, thus interfering diagnostic accuracy [51]. Another important concern is lack of standardization. There exists a high degree of variation in the sample collection, processing, storing, and the method of analysis among the different laboratories [52]. Disagreement between anticoagulants, centrifugation conditions, extraction systems, and sequencing environments may have a major impact on biomarkers output and data analysis [53].

The lack of reference impedes cross-study comparability and clinical validation. Liquid biopsy is further complicated due to biological heterogeneity. There is spatial and temporal heterogeneity in tumors resulting in changing biomarker shedding patterns with time [54]. Therapeutic evolution in clones can create varying molecular phenotypes that make it difficult to track them longitudinally and estimate response to treatment [55]. Moreover, the interaction between host microenvironment and tumor cells contributes to the release and stability of biomarkers and further complicates the situation. Clinical adoption is also limited by economic and infrastructural factors especially in low and middle-income nations. The expense of next-generation sequencing coupled with sophisticated bioinformatics and specialized machinery make them inaccessible [56]. Also, lack of trained staff as well as integrated data management system are challenges in large scale implementation. Ethical and regulatory issues are new areas of concern in the study of liquid biopsy [57]. The growing possibility to identify incidental genetic findings, germline mutations and early-stage malignancies brings up concerns of patient consent and privacy of data and its psychological implications. Therefore, a clear regulatory framework and ethical regulations are required to use these technologies responsibly. Standardized protocols, cost-effective, and effective quality control systems should be implemented in future studies [58]. Moreover, experimental in vitro and ex vivo models, including micro-mass culture and embryonic models, have been proposed to measure cellular response to toxic and environmental stressors, providing mechanistic understanding of disease processes and behavior of biomarkers. Research on such models has shown that the biochemical modulation of cellular developmental processes and responses to toxicity with antioxidant supplement can be of significant consequence [59,60]. The methods emphasize the use of controlled biological systems in the process of validating prospective biomarkers prior to clinical translation.

Advancements in the development of nanotechnology, biosensors, and microfluidics would improve detection limits, while minimizing the complexity of the assays [61]. Combination of liquid biopsy with imaging data, clinical parameters and electronic health records will further enhance precision oncology models. Also, it needs to be followed by large-scale prospective clinical trials in order to prove clinical utility, cost-effectiveness, and long-term patient outcomes [62]. The participation of collaborative consortia and international research networks will standardize methodologies and speed up translational developments. Further technological advancement, interdisciplinary cooperation, and regulatory harmonization in the next decade, are likely to turn liquid biopsy into a component of personalized cancer management instead of an adjunct diagnostic tool.

Conclusion and Future Outlook: Liquid biopsy has emerged as a revolutionary technique in contemporary oncology to provide least invasive yet dynamic access to molecular data to detect, monitor and optimize treatment of cancer. Although circulating tumor DNA has contributed to the development of this domain, there have been increasing calls to go beyond the single-analyte approach and embrace the multidimensional nature of tumor biology. Several novel biomarkers, such as circulating tumor cells, extracellular vesicles, circulating RNA, tumor-educated platelets, and circulating proteins and metabolites, can be used together to gain a better understanding of tumor heterogeneity, transcriptional activity, metastatic potential, and therapeutic resistance. Another approach that is getting considerable progress in comprehensive and personalized cancer profiling is integrative multi-analyte platforms using complex sequencing technologies and artificial intelligence-based analytics.

Even with significant advances, issues regarding analytical standardization, biological variability, cost and regulatory control still hamper large-scale clinical use. A combination of research, clinician, policymaker, and industry partners are needed in addressing these barriers. To attain equity in access and sustainable implementation, large-scale validation, infrastructure development, and training of the workforce should be approved of. In the future, further development of biosensing technologies, multi-omics technologies, and computational modeling should bring

greater accuracy and clinical utility of liquid biopsy. These approaches will provide central role in initial cancer diagnosis, real time monitoring of disease, and personalized treatment plans.

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Conflict of Interest

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