MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

LIQUID BIOPSY FOR PATIENTS WITH CANCER: DIFFERENT APPROACHES AND CLINICAL APPLICATIONS

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Abstract: Treatment of cancer patients is now based on extensive analyses of cancer cells obtained by tissue biopsy. In addition to being invasive, tissue biopsy relies on the analysis of a single cancer sample at one point in time, which may not be representative due to cancer heterogeneity and clonal evolution. Liquid biopsy is a minimally invasive test done on a sample of blood or another bodily fluid from a patient, and it has potential to overcome these limitations of tissue biopsy. Liquid biopsy has been studied as a potential diagnostic, prognostic and predictive marker in patients with cancer. Several limitations for wider application of liquid biopsy in routine clinical practice still remain, such as a lack of consensus on detection methods, an abundance of difficulties in analyzing sequencing information, and the so far limited proof of clinical utility based on large clinical trials. Three most widely studied approaches to liquid biopsy in cancer patients are the analysis of circulating tumor cells, circulating tumor DNA and exosomes. Each of these approaches has its advantages and limitations, which are discussed in this review. The focus of this review is on clinical studies analyzing the potential clinical utility of liquid biopsy in the treatment of patients with different types of cancer.

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INTRODUCTION

Traditional cancer biomarkers and imaging techniques are important in cancer diagnosis and monitoring; however, their specificity and stability through cancer progression leaves much to be desired.[1,2] The current standard of cancer diagnosis is still tissue biopsies, which represent a single point in time of a single cancer lesion. As the available techniques for the analysis of biopsies become more advanced, the limitations of tissue biopsies are becoming more evident. The inadequacies in dealing with the heterogeneous nature of the genetic profile of cancer, especially when it can change over time, make the treatment decisions based on a biopsy from a single point in time inaccurate and lacking.[3] Multiple biopsies at different points in time from the primary cancer and metastases could be a potential solution to the problem posed by cancer heterogeneity. However, multiple biopsies cause considerable discomfort and potential surgical complications for a patient, and increase the cost of cancer patient management.[4] Furthermore, there are considerable difficulties in sampling some cancer lesions due to them being inaccessible or their location obscure.[4, 5] Due to all of these issues, liquid biopsies are being considered more and more for early cancer diagnosis, monitoring of tumor progression and recurrence.[1, 6]

Liquid biopsies are minimally invasive tests that have been listed as one of the ten breakthrough technologies in 2015 by the MIT Technology Review.[7] Circulating tumor cells (CTC), circulating tumor DNA (ctDNA) and exosomes all fall under the umbrella term of liquid biopsies.[3] They all are emerging as powerful sources of diagnostic, prognostic and predictive information. Their quantification and qualitative evaluation

represent a non-invasive marker of the primary lesion and metastases.[8] With the evolution of more sensitive and specific detection methods and technologies, liquid biopsies have found many areas of application like patient stratification, screening, monitoring treatment response and detection of minimal residual disease after surgery/recurrence.[5] It should be noted that several limitations remain, such as a lack of consensus on detection methods, an abundance of difficulties in analyzing sequencing information and the so far limited proof of clinical utility based on large clinical trials.[1, 9]

CIRCULATING TUMOR CELLS

T.R. Ashworth first described epithelial cells, similar in appearance to the primary cancer cells, in the blood of a metastatic cancer patient almost 150 years ago.[10] Since then, the establishment of robust detection techniques has brought attention back to CTCs in the blood of cancer patients.[11] CTCs are shed from the primary cancer and enter the vasculature early in tumorigenesis. It is still a matter of debate if the process of releasing CTCs is a random process or a targeted occurrence. CTCs are especially important in the metastatic process in carcinomas as they may constitute "seeds" for metastatic cancer growth in distant organs.[12] They are a rare cell population in the blood, with usually fewer than 10 cells/mL (compared to the 1 million white blood cells/mL), and in their dormant state they can survive up to several years in peripheral blood. Despite this, of around 100 CTCs that enter the bloodstream daily, approximately 85% disappear within 5 minutes. Harsh conditions in the bloodstream might exert a strong selection process on the CTCs. The surviving CTCs get cleared by extravasation into the secondary organs, most notably the liver. Only 2.5% of CTCs actually cause micrometastases, and 0.01% form macroscopic metastases.[5, 8, 10, 13] To contribute to the chance of metastasis, the cancer cells form hetero-aggregates along with activated platelets to support attachment to the endothelium. Chemokine gradient is also an important factor in directing cancer cells through the vasculature.[1, 12]

CTCs that settle in the secondary organs are called disseminated tumor cells (DTCs).[14] More DTCs can be collected from the bone marrow than CTCs from the blood, but the sampling of bone marrow is a more invasive procedure. Because of their presence in peripheral blood, CTCs can be obtained using a simple venipuncture, allowing for a simple and noninvasive way to assess metastatic status, as well as to take multiple biopsies at different points in time. Resampling at different times provides for a real-time liquid biopsy. CTCs have been detected in patients months and even years after primary cancer resection, indicating CTC recirculation from secondary metastatic sites.[13, 15] Epithelial mesenchymal transition (EMT) is thought to be the process by which most of the CTCs acquire their phenotype. Those that do not express the EMT phenotype express characteristics of stem cells, which might explain their high resistance to systemic therapies and their recurrence phenomena.[8]

One of the main problems with CTC detection is their rarity. Along with better detection methods, CTC enrichment technologies have been developed to get around this issue. Enrichment techniques are immunology- or morphology-based, while detection methods are cytometric or nucleic acid-based.[1, 3, 12, 13] Morphology-based detection techniques are density gradient centrifugation and filtration by cell size. Immunology-based detection techniques can be separated into positive techniques for the selection of CTCs by immunomagnetic isolation with antibodies specific for epithelial cell adhesion molecule (EpCAM) or cytokeratin (CK), or negative techniques for the depletion of mononuclear cells by anti-CD45 antibodies. Detection techniques include immunofluorescent staining, FISH, PCR-based techniques and genomic hybridization, with PCR-based techniques being the most sensitive.[12, 13, 16]

CLINICAL APPLICATIONS OF CTCs

In 2004, CTC enumeration using the CellSearch technique was shown to be significantly associated with overall survival and progression-free survival in patients with metastatic breast cancer.[17] Changes in CTC count after the beginning of therapy were also found to be correlated with therapy outcome.[18,19]

Since then, enough research has been done on CTCs to undisputedly establish the superiority of CTC analysis over classical serum tumor markers (CEA, CA15.3) in metastatic breast cancer.[20] CTC enumeration by CellSearch has shown superiority over classical markers in prostate cancer therapy monitoring and was approved by US Food and Drug Administration (FDA) in 2008.[19]

CTC detection using the CellSearch technique is so far the only FDA-approved technique for detecting CTCs as a prognostic factor in patients with metastatic breast, prostate and colorectal cancers.[1, 6, 21, 22] Numerous studies and trials have demonstrated the prognostic value of utilizing this technique, establishing the "general guideline" of >5 CTCs per 7.5mL of peripheral blood as a prognostic factor of strong metastatic potential and an unfavorable clinical outcome.[8, 16, 23, 24] This cut-off value has been contested with recent studies suggesting a cut-off of 3 or more detected CTCs.[25, 26] More research will have to be conducted on the subject to come to a consensus on standardization practices.

The prognostic impact of bone marrow DTC with a level-of-evidence 1 has been acknowledged, and entered the 2010 TNM classification of breast cancer. There are ongoing studies and clinical trials trying to establish the CTC count using the CellSearch technique as a level-of-evidence 1 prognostic factor as well.[12, 20] The comparison of CTC count at the beginning of therapy and after therapy has shown strong evidence of its being a potential marker to guide therapy decision.[10, 23] Still, the largest trial done so far on the assessment of treatment change based on CTC

detection, S0500, showed no change in overall survival when changing therapies.[1, 22, 27] This has postponed the clinical utilization of CTCs in clinical decision making. Multiple new clinical studies have shifted focus to specific CTC phenotype detection, HER2 in particular, to guide treatment decisions.[24] This shift towards distinguishing subpopulations of CTCs seems to hold promise for a further increase in specificity and accuracy of CTC assays.[15, 16, 24, 28, 29] With advances in NGS technologies, single cell RNA and DNA analysis are becoming more viable in CTC origin and genotyping analysis, such as EGFR and KRAS expression analysis.[15, 25]

Using the CellSearch technique, it is possible to detect CTCs in patients with chronic obstructive pulmonary disease and in certain other patients with non-metastatic cancer. Monitoring patients in which the CTCs had been detected led to early detection of lung nodules and surgical resection.[12, 30]

CTC assays reach even higher specificities in terms of metastasis and tumor grade prognosis when combined with certain surface-specific molecules. When combined with CTC detection, CK19, hMAM, and EpCAM have shown 100% specificity in determining metastasis prognosis and tumor grade, providing very strong evidence for potential clinical use.[5, 8, 16, 31]

ctDNA

During apoptotic and necrotic processes, DNA is released from cells into circulation. This DNA is called cell-free DNA (cfDNA). Cell-free DNA was first described by Mandel and Metais in 1948, in the blood of healthy individuals. Circulating tumor DNA (ctDNA) is cfDNA that originates from cancer cells.[32, 33] In healthy individuals, cfDNA is almost completely cleared by the spleen, liver and kidneys, keeping its concentration low. The clearance of cfDNA is such that its half-life is about 16 minutes.[12] Elevated concentrations of cfDNA are caused by inflammation and excessive cell death which result in insufficient clearance.[1, 34, 35] Late stage cancer patients have the highest amount of cfDNA in their blood, but most of it is believed to be DNA originating from non-malignant cells and tumor stroma.[5] The variability of ctDNA amounts in cancer patients is likely associated with cancer stage, progression and vascularity.[4, 5, 34, 36]

There are both passive and active mechanisms by which DNA enters the blood circulation. Passive mechanisms are those that cause nuclear and mitochondrial DNA to enter the circulation following cellular destruction, namely apoptosis and necrosis. The active mechanism is the spontaneous release of DNA into the circulation by the cells. Active secretion of ctDNA by cancer cells has been suggested to have a signaling function.[1, 4, 33, 35] ctDNA might also have its origin in CTC cells, but since there is 17ng of cfDNA per ml of blood, and less than 10 CTCs per 7.5 ml of blood on average, there would have to be over 2000 cells per ml of plasma with the average 6pg of DNA per human cell. This means that CTCs cannot be the primary origin of cfDNA in cancer patients.[4]

So far, studies have shown blood plasma to be the optimal source for cfDNA analysis. Blood serum and plasma are both whole blood cell-free fractions. The difference between them is that serum does not contain clotting factors. Leukocyte DNA enters the cfDNA pool during clotting because of leukocyte lysis. Plasma has a much lower amount of leukocyte DNA, making it better for cfDNA analysis than serum.[5, 9, 37] cfDNA is reportedly fragmented, around 150-200bp, which is also about the size of histone DNA. Fragments shorter than 150bp have a higher prevalence of cancer-related mutations, which was shown by mutation abundance analysis with massive parallel sequencing. A comparison of mutational abundance between cfDNA and CTCs in the same patient has shown a higher abundance in cfDNA.[5, 34]

The main issue with ctDNA research is the isolation and discrimination of ctDNA from non-neoplastic DNA.[9] Precautions must be taken during ctDNA isolation to maximize the yield of isolated ctDNA and to avoid any blood cell DNA contamination.[1, 34]

Multiple sensitive methods for the detection of ctDNA have been developed, such as BEAMing, Safe-SeqS, TamSeq and digital PCR. Detection methods can be separated into targeted (detecting mutations in a set of predefined genes) and untargeted (whole-genome sequencing).[12, 34]

An additional advantage of cfDNA compared to CTCs is that it can be analyzed from frozen bio-banked biofluids.[5]

CLINICAL APPLICATIONS OF cfDNA

The search for biomarkers in the field of ctDNA has suggested multiple markers for early cancer detection, prognostics and cancer patient follow-up. Significant correlation has been found between disease stage and presence of cancer-associated mutations such as TP53, KRAS, APC and allelic imbalances in breast, pancreatic, ovarian, oral squamous-cell and colorectal cancer patient blood.[1, 4, 38] TP53, KRAS and APC monitoring in post-surgery colorectal cancer patients has shown 100% sensitivity and specificity in disease recurrence prediction,[35, 38] while MYCN amplification has been associated with poor outcome.[1, 39] The amounts of ctDNA found in patients were also correlated with disease progression and survival.[1, 35]

cfDNA detection methods have come a long way, with commercial PCR kits using LINE1 and ALU repeats to determine cfDNA size readily available. Distinguishing ctDNA from cfDNA requires the presence of tumor-related mutations, such as mutations in the RASSF1A gene. With progress made in methods and techniques, certain research groups have reached detection levels of 0.01% of mutants present in wild type cfDNA, with clinical trials being under way.[1, 6]

Recent studies have shown a potential attractive application of ctDNA in the clinical management of cancer patients. It has been shown in breast cancer that a ctDNA assay on TP53 and PIKC3A mutations has higher specificity in detecting metastatic disease than the classic CA15-3 marker or a CTC assay.[35-37, 40] The

detection of losses of heterozygosity at the tumor suppressor genes TIG1, PTEN, cyclin D2, RB1 and BRCA1 on ctDNA was associated with a more aggressive biology of breast cancer.[35, 41] Enough research has been done on some of the suggested biomarkers for comprehensive meta-studies to stress their potential clinical applications. PIKC3A is one of them, with the diagnostic accuracy of PIKC3A mutation detection by ctDNA analysis being high enough for potential clinical application.[8, 42]

A potential biomarker has been found in the ctDNA methylation status. RASSF1A, APC and DAP kinase were found hypermethylated in patients with benign lesions and carcinoma *in situ*. Their methylation status was correlated with worse prognosis.[8, 35, 43]

The detection of genetic mutation in tumor DNA is used in guiding clinical decision-making for multiple different therapies like EGFR mutations for gefitinib in NSCLC, BRAF mutations for vemurafenib in melanoma, ESR1 mutations for the non-efficiency of fulvestrant in breast cancer or KRAS mutations for cetuximab and panitumumab in colorectal cancer. It is noteworthy that the same genetic alterations are detectable in ctDNA.[4, 9, 44-46]

Studies have shown that successful therapy monitoring of acquired resistance as either an increase of gene copy number (in the case of BRAF or MET in melanoma and lung cancer) or a de novo resistant mutation is possible with ctDNA analysis.[4, 9, 46-48]

EXOSOMES

Exosomes are a class of extracellular vesicles between 50 and 150 nm in diameter. They are formed during the inward budding of endosomes when nucleic acids and proteins are encapsulated inside them. Finally, exosomes are released into the extracellular space and enter the circulation.[1, 3, 49, 50] The term exosomes was first used in 1981 to describe membrane-enclosed structures released from the surfaces of cultured cells.[51]

Exosomes are released by various cell types such as immune cells, platelets, endothelial cells and cancer cells.[49] Research suggests that cancer cells release more extracellular vesicles than non-cancer cells and that their protein concentration is higher, which might be due to response to a number of oncogenes. They have been found circulating in the blood, urine, cerebrospinal fluid and ascites of both healthy individuals and cancer patients.[1, 50] Exosomes seem to function as intercellular messengers.[3, 5] In cancer growth and progression, their main functions seem to be promotion of angiogenesis, tissue invasion, and suppressing the host immune response.[50, 52, 53]

Many commercial kits for exosome isolation as well as characterization and isolation protocols have been developed, making the otherwise common problem of isolation in liquid biopsy less of an issue in exosome research.[54] The methods used for isolation are ultrafiltration with size exclusion chromatography, precipitation with polymers and immunoaffinity purification with magnetic beads[1] Exosomes express specific markers such as HSP70 and Alix, allowing for simple separation from other subcellular vesicles. They also contain surface markers from their cells of origin, making enrichment strategies possible.[5, 53]

Exosomes are of particular interest in liquid biopsy research due to the fact that they contain cancer-specific proteins and RNA, shielded from the proteinases and RNases in the circulation by the membrane.[1, 5, 54] Current research has shown that cancer cells actively release tens of thousands of exosomes per day, which translates into hundreds of billions of vesicles per mL of plasma. That, coupled with the fact that exosomes are stable in the circulation, means that cancer-specific proteins and RNA can be isolated in abundance and stored for years.[5] This opens the prospect of easier mutational and expressional cancer RNA analysis.[3]

An issue with exosome research is that most conventional cancer-associated markers are not specific to cancer-derived exosomes. Identification and isolation of cancer-specific exosomes without contamination from non-cancer exosomes is something that needs more attention.[49] However, since exosomes are a source of cancer cell proteins and nucleic acids they are promising targets for the identification of cancer-specific markers with the rapid advances in technologies, especially NGS for RNA and mass spectrophotometry for proteins.[1, 50]

CLINICAL APPLICATIONS OF EXOSOMES

Research on cancer-derived exosomes has found Glypican1 (GPC1) to be a potential pan-cancer exosomal marker. In particular, isolation of GPC1 expressing circulating exosomes (crExos) has identified KRAS mutations with 100% correlation to the ones in the cancer tissue. Studies done on mice models have suggested that GPC1 exosomal concentration could be used to distinguish malignant from benign disease, and carcinoma *in situ* from advanced carcinoma stages.[1, 50] This combination is most often found in pancreas cancer cell lines, but it is abundant in other tumor types as well, showcasing its potential as a pan-cancer marker.[49]

Studies done on exosomal miRNA and proteins have shown several potential prognostic and diagnostic markers. MiR-718 has been shown as a risk factor for recurrence after liver transplantation, miR-92a downregulation was associated with cancer progression and disease recurrence in hepatocellular carcinoma, while overexpression of miR-21-3p indicates cisplatin resistance in ovarian cancer.[55-57] MiRNA-10b, miRNA-21, miRNA-122 and miRNA-200a levels were found to be drastically changed in cirrhosis and hepatocellular carcinoma[58] while miR-29a and miR-21 were found to be increased only in the presence of breast malignancies[59] A high expression of migration inhibitory factor in pancreatic ductal adenocarcinoma exosomes may represent a prognostic factor for the development of metastases.[60] Overexpression of miR-105 in breast cancer exosomes could differentiate between low and high metastatic cells lines.[61] MiR-1246, miR-3976, miR- 4644 and

miR-4306 have been upregulated in 83% of pancreatic adenocarcinomas,[62] while miR-125b downregulation has been shown to correlate with disease progression.[54,63] Studies have also shown the potential of survivin as marker for early cancer detection and response to treatment.[52] MiR-320 and miR-574-3p along with RNU6-1 could serve as diagnostic biomarkers for the detection and monitoring of glioblastoma.[64] Cd24+ exosomes have been reported as potential markers for diagnosing ovarian cancer.[65] These markers have so far been studied on cell lines or small cohorts of patients, so more research of larger cohorts of patients is needed to validate their clinical use.

Studies on potential therapeutic uses of exosomes have come a long way. It has been shown that miR-134-loaded exosomes can decrease migration and invasion of breast cancer cells and exosomes loaded with miR-503 can inhibit proliferation and invasion of breast cancer cells.[54, 66] Engineered exosomes (known as iExosomes) have shown much promise. A study on mice has shown that iExosomes engineered to carry siRNA specific for oncogenic G12D mutated KRAS can efficiently target oncogenic KRAS and suppress pancreatic cancer.[67] An *in vivo* study done on mice using exosome to deliver let-7a to epidermal growth factor receptor expressing breast cancer cells has further pointed to their validity as therapy carriers. Studies have identified certain milk proteins which help exosomal delivery.[54, 68]

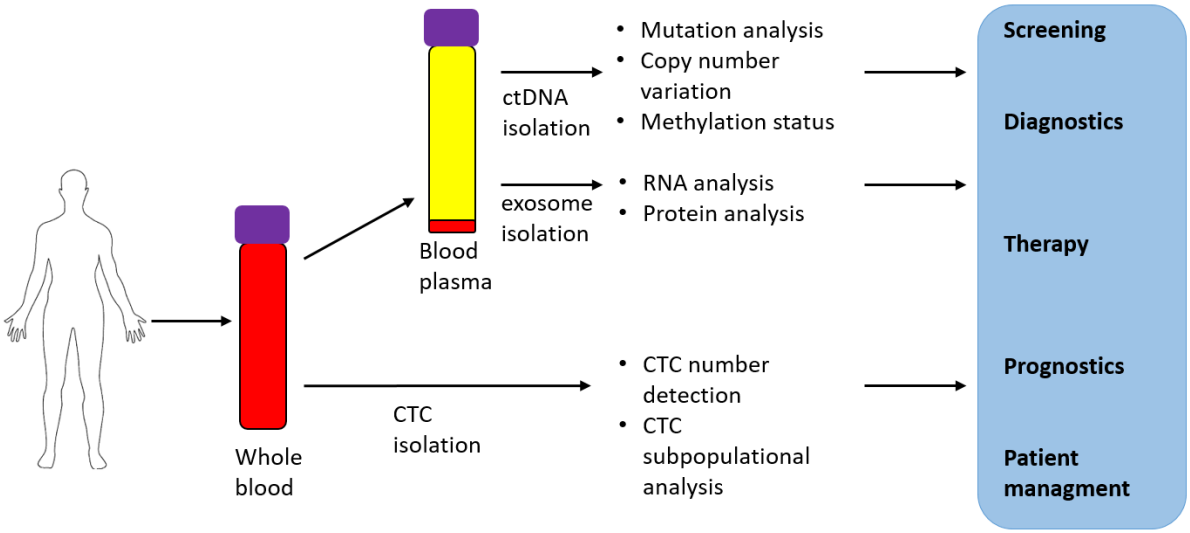

Figure 1. A possible liquid biopsy workflow. The diagram showcases the ease of use of liquid biopsies and some of the possible analyses conducted on different liquid biopsy samples. Some of the possible uses of liquid biopsies are highlighted.

CONCLUSION

Liquid biopsies are an important part of the precision medicine field related to its goals of noninvasive procedures that can be tailored to the patient's unique needs (Figure 1).

The diagram showcases the ease of use of liquid biopsies and some of the possible analyses done on different liquid biopsy samples. Some of the possible uses of liquid biopsies are highlighted.

As such, research on liquid biopsies has had a strong start in the field of cancer diagnostics, with potential to drastically reduce invasive and potentially harmful procedures and cut economic costs. However, specificity and sensitivity issues still plague the potential for early detection of cancer. The use of liquid biopsies for monitoring during systemic therapy and for detection of mutations responsible for resistance to targeted therapies has shown greater progress, and is closer to introduction in clinical practices.

The main issues are still a lack of standardized techniques, especially with ctDNA and exosome isolation, which can lead to a myriad of downstream issues. The selection of tumor markers is also a contested point, since distinguishing between CTCs with high and low metastatic capacity and exosomes and cfDNA derived from tumor and normal tissues remains problematic. The advances in technology (NGS in particular) and increasing attempts at standardization and clinical practice recommendations show promise in this regard.

More randomized clinical trials have to be done on the clinical utility of liquid biopsies, since most of the studies conducted as of yet have been retrospective and have provided little evidence of both clinical validity and utility for widespread use of liquid biopsies. The increasing use of liquid biopsies assay in clinical care is likely to provide the evidence needed.

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