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Liquid biopsies: tumour diagnosis and treatment monitoring

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Received: 28 May 2016 / **Accepted:** 01 Aug 2016 / **Published online:** 30 Aug 2016

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Abstract— Cancer is a disease with high evolutionary, i.e., malignant, characteristics that change under selective pressure from therapy. Characterization based on molecular or primary tumor properties or clinicopathological staging does not fully reflect the state of cancer, especially when cancer cells metastasize. This is the major reason for failure of cancer treatment. Currently, there is an urgent need for new approaches that allow more effective, but less invasive, monitoring of cancer status, thereby improving the efficacy of treatments. With recent technological advances, “liquid biopsies,” the isolation of intact cells or analysis of components that are secreted from cells, such as nucleic acids or exosomes, could be implemented easily. This approach would facilitate real-time monitoring and accurate measurement of critical biomarkers. In this review, we summarize the recent progress in the identification of circulating tumor cells using new high-resolution approaches and discuss new circulating tumor nucleic acid- and exosome-based approaches. The information obtained through liquid biopsies could be used to gain a better understanding of cancer cell invasiveness and metastatic competence, which would then benefit translational applications such as personalized medicine.

Keywords: Circulating tumor cells, circulating tumor DNA, circulating tumor RNA, exosomes, liquid biopsy

INTRODUCTION

While most studies of cancer treatment aim at improving the efficiency of killing cancer cells, the question is whether the disease can be prevented early, or whether malignant cells can be detected early enough to employ effective methods in reversing the disease process. There are clearly no simple answers to the above questions because so far, we have only known some of the factors that predetermine an individual's cancer risk, such as abnormalities on BRCA1/2 associated with breast or ovarian cancer. This is also the basis for determining the presence of serum proteins related to cancer status, such as carcinoma antigen-125 (CA-125), carcinoembryonic antigen (CEA), or prostate-specific antigen (PSA). However, there is evidence indicating that these protein marker-based methods are not enough because they are also found in low concentrations in

healthy individuals. This presents the question, how early can cancer be detected? Early and proper cancer detection can improve prognosis by allowing early intervention when malignant cells are gradually forming a tumor. Tumors detected by the current image capture techniques already contain over 10 billion cancer cells, indicating that they have already micrometastasized to other locations. This results in cancer persistence; the high proportion of malignant cells resistant to therapy can cause relapse and increase metastatic risk, ultimately increasing mortality rate. Evidence shows that cancer is closely related to genetic alterations, including substitution, insertion, deletion, or translocation, that confer gene fusion, amplification, or loss of heterozygosity. The detection and identification of specific sources containing mutated genes would pave a more effective way to cancer screening and monitoring. Liquid biopsy can assess a patient's oncogenic

mutation profile, which is frequently associated with cancers such as epidermal growth factor receptor (EGFR) and KRAS (Tu et al., 2016). While this approach focuses on biopsies acquired from tumor locations determined by the current imaging procedures, it is greatly hampered because: (a) certain types of non-solid tumors can only be obtained by fine-needle aspiration; (b) even if biopsies are collected, this procedure is highly invasive, causing serious consequences, including risk to patients and significant cost, making repeated testing to monitor disease status difficult; (c) only a small amount of cytological material can be obtained, which is insufficient for genetic analysis; (d) tissue preservation methods such as formalin fixation are needed, increasing the risk of modifying genetic components, specifically deamination of cytosine (C to T transitions); (d) tumor tissue has very high heterogeneity since cancer cells can mutate under the selective pressure of cytotoxic therapies, the reason why biopsies do not fully reflect the characteristics of the tumor. Detection and characterization of genetic material in blood has been garnering considerable attention. Among the alternative sources that are more informative and representative of the tumor, the most prominent candidates are intact circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA), and circulating cell fragments (exosomes). While CTCs are derived from primary or metastatic tumors, ctDNA is released from lysed CTCs. In case biopsies are not available, CTCs and ctDNA can be perfect tools for the rapid identification and noninvasive clinical monitoring of cancers, as well as for devising effective treatment strategies. The genetic variations in CTCs and ctDNA fully reflect the tumor status and immediately respond to targeted inhibitors. Tumors have high heterogeneity, which leads to each patient responding differently to the same treatment. Applying treatment that is not based on the targeted diagnosis puts the cost burden on patients and causes unnecessary side effects during the treatment (Pachmann et al., 2011).

CIRCULATING TUMOR CELLS (CTCs)

During cancer progression, malignant cells acquire resistance to targeted drugs through a variety of mechanisms, such as: (i) stopping the signals passing through the receptor affected by the drug molecules

by activating alternate signaling pathways and ensuring proliferation of the tumor; (ii) increasing expression of antiapoptotic transcription factors; or (iii) changing the cellular phenotype (i.e., cell transformation) through epithelial-to-mesenchymal transition (EMT). EMT facilitates another complicated process, metastasis, which includes a series of successive events. One of the first events in metastasis is intravasation of circulating tumor cells (CTCs), which originate from highly aggressive cells that have acquired increased migratory potential or from abnormal blood vessels that supply the tumor.

In breast cancer, an increase in CTCs is concurrent with a significant increase in HER2/neu gene amplification (Pachmann et al., 2011). Understanding the biology of CTCs will help in early detection and monitoring of tumor status, as well as in establishing an effective targeted therapy to prevent metastasis. We can: (i) get information to estimate the risk of metastasis, (ii) monitor targeted therapies in real-time, (iii) discover new potential targets, and (iv) identify mechanisms responsible for resistance and metastatic progression (Alix-Panabieres and Pantel, 2013a). However, isolation of intact CTCs is the proverbial “needle in a haystack,” as it is a daunting task to find an extremely rare cell in a “sea” of normal cells circulating in the blood. Some studies have reported hundreds or even thousands of CTCs/ml of blood, but most of the analyzed samples have yielded less than 10 cells/ml. As a reference, 1 ml of whole blood contains over 1 million white blood cells and more than 1 billion red blood cells. Clearly, isolation of CTCs needs complex platforms that can: (a) target the physical properties of tumor cells, providing size-based filtering, size-based flow kinetics, differential density, and electrical charge or photoacoustic resonance; (b) target the expression of unique cell markers, allowing staining of tumor surface markers or secretion marker proteins; and (c) exclude normal cells and select cells that can invade coated surfaces.\

Although significant progress has been made in CTCs isolation, the above methods are questionable with respect to accuracy. In case of the method based on physical properties, namely size-based filtering, not all epithelial cancer cells are larger than leukocytes. The method based on the expression of the EpCAM marker to identify cancer cells has a risk of missing EpCAM-negative CTCs (which undergo EMT) and cannot be applied to non-epithelial carcinoma (such as sarcoma). In addition to the obstacles presented by the

number of rare cells, the difficulty of cell acquisition, high heterogeneity of the tumor and the diversity of distant metastases, the lack of a suitable sample for comparing results is another major drawback of CTC analysis. Lin et al. used a microfluidic mixer to coat CTCs with a large number of microbeads to amplify their size and enable complete discrimination from leukocytes (Lin et al., 2013).

Because tumors are very dynamic, discovery and validation of novel CTC markers expressed on mutated, or rarely on normal cells, is essential. However, up until now, the task has been in its infancy. CTCs have a short half-life, usually of a few hours, and are difficult to store, making delayed analysis almost impossible. CTC assays require the selection of appropriate methods to increase the number of cells up to many folds (enrichment step) for further analysis. Without the careful selection of specific cancer cell markers, there is high possibility of false-positive or false-negative identification, separation, and characterization of CTCs. Markou et al. developed a multiplexed PCR-coupled liquid bead array to enrich CTCs and detect the expression of six CTC genes, including keratin (19KRT19), Erb-B2 receptor tyrosine kinase 2 (ERBB2), secretoglobin (SCGB2A2), melanoma antigen family A (MAGEA3), twist homolog 1 (TWIST-1), and hydroxymethylbilane synthase (HMBS), making it possible to detect the expression of each gene at single-cell level (Markou et al., 2011). Measuring androgen receptor signaling and levels of prostate-specific antigen and prostate-specific membrane antigen in CTCs helps guide therapy in metastatic prostate cancer (Miyamoto et al., 2012; Pantel and Alix-Panabieres, 2012; Stott et al., 2010).

CTCs can replace tumor biopsies for predicting tumor recurrence and guide effective therapeutic management (Cai et al., 2014). In non-small cell lung cancer (NSCLC), mutations involving EGFR-encoding gene are frequent; therefore, assessing EGFR mutations present in CTCs may provide real-time information on disease status. Marchetti et al. showed that preparations of CTCs obtained by the Veridex CellSearch System, coupled with ultra-deep next-generation sequencing (NGS), could be a sensitive and specific diagnostic tool for optimization of pharmacologic treatment (Marchetti et al., 2014). The EPithelial ImmunoSPOT technology has been currently used to detect viable CTCs at single-cell level, and has been employed with different tumor

types, including breast, prostate, and colon cancer, as well as melanoma (Alix-Panabieres and Pantel, 2015).

For CTC-based research to translate to clinical utility, CTC analysis must allow serial evaluation of patients most likely to benefit from targeted drugs developed based on disease characterization at the molecular level.

The developing technologies for CTCs analysis facilitate the discrimination of molecular subtypes of the disease and distinguishing genetic variation over time (Cortesi et al., 2015). Liquid biopsy has gradually become a fingerprint for individual tumors, making it possible to track evolution of cancer at every stage. An intact CTC contains DNA, RNA, and protein for wide and deep analysis; moreover, CTCs could be expanded *in vitro* to a sufficient number to enable investigation into the features of metastases-initiating cells (Gazzaniga et al., 2015). The advent of new sensitive technologies permits the isolation of rare CTCs from the blood, making it possible to explore the clinical utility of these cells as prognostic and pharmacodynamic biomarkers in many solid tumors, including lung cancer (Zhang et al., 2015).

Bone marrow (BM) is a frequent site of metastasis in various types of epithelial tumors, including breast, colon, lung, prostate, esophageal, gastric, pancreatic, ovarian, and head and neck cancer. It has been shown that cancer cells with increased metastatic-potential, called disseminated tumor cells (DTCs), are present in BM. In addition to CTCs, DTCs in BM may provide important insight into the biology of cancer metastasis. The detection of DTCs at single-cell level has been made available with the developing immunocytological and molecular methods (Pantel and Alix-Panabieres, 2014).

Microfluidic chip-based micro-Hall detector (muHD), which has the high bandwidth and sensitivity of semiconductor technology, can detect single CTCs in whole blood, allows for high-throughput screening, and identifies a panel of biomarkers, such as EpCAM, HER2/neu, and EGFR, on individual cells (Issadore et al., 2012).

Cytokeratin-19 (CK-19) has a crucial role in maintaining epithelial cell morphology. The release of full-length CK-19 by human tumor cells is an active process, and is the reason why full-length CK-19 detection is considered a marker of viable tumor cells and of early metastatic progression. Alix-Panabieres et al. performed EPISPOT (EPithelial ImmunoSPOT)

assays to analyze the release of full-length CK19 in colorectal and breast cancer cell lines. The biology of CK19-release was further analyzed with mass spectrometry, cycloheximide, Brefeldin A, and vincristine. The results showed that CK19-EPISPOT was more sensitive than CK19-ELISA. This incidence and number of CK19-releasing cells (RCs) were correlated to overt metastases and reduced patient survival (Alix-Panabieres et al., 2009). The author also applied a novel ELISPOT assay (designated "EPISPOT"), which detects viable CTC/DTC protein fingerprint from single epithelial cancer cells, for CK19 and mucin-1 (MUC1) in breast cancer, and fibroblast growth factor-2 (FGF2) in prostate cancer (Alix-Panabieres, 2012).

Somlo et al. used a multiple biomarker assessment, which simultaneously quantifies the expression of HER2, estrogen receptor (ER), and ERCC1 (a DNA excision repair protein), as well as novel fiber-optic array scanning technology (FAST), for sensitive localization of CTCs (Somlo et al., 2011). Ntourioupi et al. used density gradient centrifugation and filtration to isolate CTCs in peripheral blood of prostate, colorectal, and ovarian cancer patients, and labeled CTCs with monoclonal antibodies against cytokeratins 7/8, and either anti-EpCam or anti-PSA. The samples were analyzed with the Ikoniscope robotic fluorescence microscope imaging system. The results showed that the sensitivity of this method could detect less than one epithelial cell per milliliter of blood, and fluorescence in situ hybridization (FISH) could identify chromosomal abnormalities in these cells (Ntourioupi et al., 2008).

Andreopoulou et al. compared the CellSearch system and AdnaTest BreastCancer Select/Detect, for isolation and characterization of CTCs in peripheral blood (PB). AdnaTest used RT-PCR to detect gene transcripts of tumor markers (GA733-2, MUC-1, and HER2). The results indicated that AdnaTest has a sensitivity equivalent to that of the CellSearch system (Andreopoulou et al., 2012). Kim et al. used a telomerase-specific replication-selective adenovirus to detect CTCs based on the principle that the adenovirus can replicate only in telomerase-expressing cells and emit fluorescence in transfected cells. The adenovirus-based assay is comparable to the CellSearch assay but provides more biological characteristics of collected CTCs than does the CellSearch assay (Kim et al., 2011).

Eifler et al. used leukapheresis, elutriation, and fluorescence-activated cell sorting (FACS) to enrich and isolate CTCs with high efficiency and purity for further molecular analysis. Tumor cells isolated using this sequential process are carboxyfluorescein succinimidyl ester positive, EpCAM positive, and CD45 negative (Eifler et al., 2011). Saucedo-Zeni et al. functionalized a structured medical Seldinger guidewire (FSMW) with an EpCAM-directed chimeric monoclonal antibody to isolate CTCs from peripheral blood of breast cancer and non-small cell lung cancer (NSCLC) patients. The FSMW successfully enriched CTCs across all tumor stages with no adverse effects (Saucedo-Zeni et al., 2012).

CIRCULATING NUCLEIC ACIDS (ctNAs)

The presence of circulating, cell-free nucleic acids (ctNAs) in blood has been described since the middle of the last century (Leon et al., 1977; Mandel and Metais, 1948; Stroun et al., 1989). Most healthy individuals (over 90%) have a small amount of cell-free DNA ([cfDNA] 25 ng/ml whole blood). cfDNA has been shown to shed from normal cells during cell replacement, apoptosis, and necrosis. However, in healthy individuals, the rate of cell replacement is low, and cfDNA is actively excreted from blood by liver and kidneys, which maintains low cfDNA concentrations. Inflammation, exercise, tissue injury, surgery, or pregnancy increases cfDNA levels up to many folds. Increased levels of cfDNA in the blood of pregnant women are derived from the fetus; therefore, one of the first applications of cfDNA was to identify antenatal prognosis. Importantly, cancer patients are also reported to show a sharp increase in cfDNA levels compared with those in healthy individuals; with very high volatility depending upon the status of cancer, ctDNA can even account for over 10% of total cfDNA. cfDNA are relatively small fragments, typically 160-180 bp in length. As mentioned previously, cancer cells accumulate genetic alterations, including point mutations and changes in structure, with many copies released and easily detected in blood through ctDNA.

As in the case of CTCs, the blood contains much larger amounts of normal cell-derived cfDNA than ctDNA, and the stability of ctDNA is challenged by the presence of DNase, which makes the half-life of ctDNA only a few hours. However, isolation of

ctDNA is a much simpler process that does not need special equipment even with small blood volumes (5-10 ml anticoagulated blood). ctDNA in plasma is used more often than ctDNA in serum to avoid contamination by genomic DNA from lysed cells. The advantages of rapid, economic, and reliable ctDNA analysis open up the prospect of high-throughput assays. A further advantage of using cfDNA instead of CTCs is that cfDNA can be analyzed from frozen biofluids, which allows for the extended storage of cfDNA. Methylation-specific real-time polymerase chain reaction of circulating tumor DNA showed that percutaneous liver biopsy does not affect hematogenous dissemination of hepatocellular carcinoma (Yu et al., 2004).

During cfDNA isolation, it is important to note: (i) blood collection and extraction protocols should avoid affecting the number and size cfDNA or contamination with wild-type cfDNA liberated from lysed leukocytes; (ii) ctDNA released by large amounts cfDNA from chemotherapy, radiation therapy, surgery or infection could increase false-positive results; (iii) genes in cfDNA analysis are normally present at equal levels. To address this last problem, the combining cfDNA and cell-free RNA (cfRNA) has been attempted, and the approach provides the advantage of detecting rare mutations. If cfDNA represents the cell death, cfRNA, which includes mRNA and non-coding RNA (microRNA, lncRNA, etc.), represents active cells because they are transcripts of highly expressed genes (thousands copies/cell) (Ono et al., 2015). Sestini et al. demonstrated that circulating microRNA increased the specificity of low dose computed tomography (LDCT) in lung cancer screening (Sestini et al., 2015).

Technological advances have been able to detect cancer-associated alleles in cfDNA released from tumor cells. Identification of both genetic and epigenetic aberrations in ctDNA could provide the genetic landscape of both primary and metastatic lesions and systematically track genomic evolution for diagnostic, prognostic, and treatment purposes (Crowley et al., 2013). Molecular analysis plays a key role in the management of malignant tumors. Tumor DNA obtained from circulating tumor DNA overcomes the limitation of static molecular or tissue biopsies. Moreover, repeated sampling of ctDNAs combines the inter- and intra-metastatic molecular heterogeneity and provides the molecular and genomic information that is similar to sampling of

tumor tissue (Nannini et al., 2014). Lebofsky et al. performed de novo detection of somatic mutations using cell-free tumor DNA (ctDNA) in plasma and compared it with biopsies of metastases across multiple types of tumors. The results show that ctDNA analysis can potentially replace the costly, harmful, and lengthy process of metastatic tissue biopsy (Lebofsky et al., 2015).

With the recent developments in sequencing and digital genomic techniques, ctDNA analysis is a step ahead of current clinical and radiological techniques in providing information for personalizing patient therapy. The applications for ctDNA are diverse and include identifying genomic alterations, monitoring treatment responses, unraveling therapeutic resistance, detecting metastasis-specific mutations, and quantifying tumor burden (De Mattos-Arruda and Caldas, 2015). Tumor heterogeneity, clonal evolution, and selection from systemic treatment result in almost all tumors becoming resistant to therapy. ctDNAs can help obtain the genetic follow-up data for categorizing tumors for clinical decisions (Heitzer et al., 2015).

K-ras mutations have been commonly found in pancreatic cancer. Kinugasa et al. compared results from DNA obtained by endoscopic ultrasound-guided fine-needle aspiration biopsy and ctDNA evaluation by digital polymerase chain reaction. They showed that K-ras mutation rates in tissue and ctDNA were 74.7% and 62.6%, respectively, with a concordance rate of 77.3%. Moreover, K-ras mutations in ctDNA was found to be associated with significantly shorter patient survival (Kinugasa et al., 2015). ctDNA has become a potential “real-time” biomarker that provides useful data before and during treatment as well as throughout cancer progression. However, there is still no standard or an accurate biomarker because cancer is an extremely complex disease. Different methods of detecting and processing ctDNA also contribute to inconsistent results. Additionally, there is still controversy as to which assay has the appropriate sensitivity and specificity for ctDNA analysis (Ma et al., 2015).

Genetic aberrations in the androgen receptor (AR) are present in castration-resistant prostate cancer. Targeted next-generation sequencing has broad clinical utility to plasma DNA. Romanel et al. sequenced plasma samples from patients with castration-resistant prostate cancer who had been treated with abiraterone, and detected a sufficiently

high fraction of tumor DNA to quantify AR copy number state. Patients with gains in AR copy numbers or AR amino acid changes had a significantly worse overall and progression-free survival (Romanel et al., 2015; Schweizer and Antonarakis, 2015).

Schwaederle et al. used next-generation sequencing (NGS) to detect and monitor alterations in circulating tumor DNA (ctDNA) in plasma extracted from patients with a variety of cancers. The results of ctDNA analysis showed that the majority of diverse cancers had detectable ctDNA aberrations; the most frequent alterations were tumor protein p53 (TP53), followed by EGFR, MET, PIK3CA, and NOTCH1 (Schwaederle et al., 2016). Conventional methods for the isolation of ctDNA from plasma are costly, time-consuming, and complex. To counter these disadvantages, Sonnenberg et al. used an AC electrokinetic device to rapidly isolate ctDNA from a drop of blood. The AC electrokinetic device separates ctDNA into dielectrophoretic (DEP) high-field regions; then, the concentrated ctDNA is detected by fluorescence and eluted for quantification, PCR, and DNA sequencing (Sonnenberg et al., 2014).

Devonshire et al. evaluated ctDNA extraction efficiency, fragment size bias, and quantification in a study that compared different methods for ctDNA extraction; the study found that analysis and averaging of multiple reference genes using the GeNorm approach provides more reliable results (Devonshire et al., 2014). Breitbach et al. conducted a direct quantitative real-time PCR (qPCR) to amplify multi-locus L1PA2 sequence for the measurement of cfDNA from plasma without previous DNA extraction. The analyses revealed higher cfDNA concentrations in unpurified plasma compared with those of the QIAamp DNA Blood Mini Kit or with those of a phenol-chloroform isoamyl (PCI) based DNA extraction (Breitbach et al., 2014).

McBride et al. mapped genomic rearrangements in solid tumors and showed that the assays could detect a single copy of the tumor genome in plasma without false positives, which paves a way to serial assessment of disease status, drug responsiveness, and incipient relapse (McBride et al., 2010). Church et al. performed duplicate real-time PCRs of circulating methylated SEPT9 DNA (mSEPT9) for detecting colorectal cancer (CRC) and found that the CRC signal in the blood can be detected in asymptomatic individuals with average risk (Church et al., 2014).

Chan et al. used Epstein-Barr virus (EBV) DNA isolated from plasma for nasopharyngeal carcinoma (NPC) surveillance in individuals who were not clinically diagnosed with NPC. Moreover, repeating the test could avoid false-positive results (Chan et al., 2013). Diehl et al. applied a highly sensitive approach to quantifying ctDNA in plasma samples from patients undergoing multimodal therapy for colorectal cancer and found that ctDNA measurements could be used to reliably monitor tumor dynamics (Diehl et al., 2008).

Spindler et al. used quantitative PCR method to assess the number of cfDNA alleles, as well as Kirsten rat sarcoma viral oncogene homolog (KRAS) and BRAF mutation alleles, in plasma from patients with metastatic colorectal cancer (mCRC) undergoing treatment with cetuximab and irinotecan. The majority of KRAS mutations detected in tumors were also found in the plasma, and cox analysis confirmed the prognostic importance of both cfDNA and pmKRAS (Spindler et al., 2012). Taly et al. investigated using multiplex picodroplet digital PCR (dPCR) to screen for the most common mutations in codons of the KRAS oncogene in the plasma of patients with metastatic colorectal cancer. The study showed that the higher sensitivity of this assay can screen for multiple mutations simultaneously in ctDNA (Taly et al., 2013).

Newman et al. coupled deep sequencing (CAPP-Seq) with broad analysis of multiple classes of somatic alterations and identified mutations in >95% of non-small-cell lung cancer (NSCLC) samples (Newman et al., 2014). Kinde et al. described an optimized approach to massively parallel sequencing, called the Safe-Sequencing System ("Safe-SeqS"), for identifying mutations in ctDNA (Kinde et al., 2011).

Based on a benchtop high-throughput platform, the Illumina MiSeq instrument, Heitzer et al. explored whole genome sequencing of plasma DNA to scan tumor genomes of patients with prostate cancer. The results revealed multiple copy number aberrations and novel chromosomal rearrangements. The approach got valuable results in distinguishing castration-resistant (CRPC) and castration sensitive prostate cancer (CSPC), and provided specific genomic signatures within 2 days (Heitzer et al., 2013). Forshew et al. developed a method for tagged-amplicon deep sequencing (TAM-Seq) and identified cancer mutations present in ctDNA at allele frequencies as low as 2% with sensitivity and

specificity of >97%. In patients with advanced ovarian cancer and metastatic breast cancer, TAm-Seq was able to identify mutations throughout TP53 and EGFR and tracked concomitant mutations (Forsheew et al., 2012).

Aliyev et al. demonstrated the utility of thyroid-stimulating hormone receptor messenger RNA (TSHR mRNA) as a marker of tumor aggressiveness in patients with papillary thyroid microcarcinoma (PTmC) (Aliyev et al., 2015). Kopreski et al. showed that 5T4 mRNA, which is well-known as a trophoblast glycoprotein frequently overexpressed in epithelial malignancies, was reproducibly detected in patients with advanced breast cancer or non-small-cell lung cancer (Kopreski et al., 2001). Rabascio et al. found that among various angiogenesis markers, circulating VE-cadherin (VE-C) RNA was increased in hematological malignancies (Rabascio et al., 2004).

Yamashita et al. conducted reverse transcriptase-polymerase chain reaction of carcinoembryonic antigen messenger RNA, which is defined as the independent prognostic factors for survival, in patients with non-small cell lung cancer who underwent a curative lobectomy (Yamashita et al., 2002). Zhou et al. showed that abnormal metabolic rate of tumor cells is responsible for the increased level of circulating RNA (Zhou et al., 2008).

CTCs vs. ctNA

Analysis of gene mutations on CTCs and ctDNA contributes to clinical management of drug resistance in cancer patients (Alix-Panabieres and Pantel, 2013b). Pantel and Alix-Panabieres showed that there are different genomic characteristics between distant metastases and the corresponding primary tumor. Moreover, at different sites, metastases show considerable intra-heterogeneity. These limitations can be solved by complementary technologies using CTCs and ctDNA in parallel (Pantel and Alix-Panabieres, 2013; Tsujiura et al., 2014).

The presence of EGFR mutations predicts poorer outcomes for patients with non-small-cell lung carcinoma (NSCLC). However, most NSCLC are not available to collect surgery specimens. CTCs and ctDNA released into the peripheral blood from metastatic deposits is an emerging strategy for NSCLC genotyping (Fenizia et al., 2015). Recently, EGFR mutations in urine and saliva samples have been

detected with simpler techniques (Lin et al., 2015). CTCs are now validated in breast, colon, and prostate cancer, and ctDNA can be used to encompass the spectrum of mutations present in tumors (Gingras et al., 2015).

Cutaneous melanoma has one of the highest incidence rates with low overall survival despite the advent of new therapeutics. It is believed that this cancer could be treated more effectively and at a lower financial burden to patients. Blood-based biomarker approaches, which exploit CTCs and cell-free circulating tumor nucleic acids (ctNAs), allow for regular dynamic monitoring of the disease and show potential in the development of individualized therapy. With advancements in improving molecular assays, such as massive parallel sequencing (MPS), liquid biopsy analysis would improve the treatment and outcomes for cancer patients (Huang and Hoon, 2015). Liver cancer is one of the top causes of cancer-related death worldwide. Most patients are diagnosed at late stages; the only treatment that improves survival in advanced disease is sorafenib. Analysis of circulating cancer byproducts could provide molecular information about the tumor, improve patient stratification, and play a role in the management of tumor over time (Labgaa and Villanueva, 2015).

CTCs and ctDNA drawn from peripheral blood, or tumor DNA in the saliva of patients with head and neck cancer, could signify early signs of the disease and present an opportunity for clinical intervention (Schmidt et al., 2016).

EXOSOMES

Analysis of CTCs provides insights into cellular components, including DNA, RNA, and protein, while ctDNA is easy to isolate and is present at higher levels than CTCs; combining these two methods can be synergistic for precise measurement of cancer heterogeneity. However, as mentioned previously, the half-life of CTCs and ctDNA is relatively short; therefore, isolation should be conducted soon after collection. A more stable source of material from the tumor is exosomes. Exosomes are inter-cellular messengers with a size range of 30-200 nm that serve multiple critical biologic functions including cellular remodeling and regulation of immune function (Santiago-Dieppa et al., 2014). Cancer cells are

reported to have increased production of exosomes, which have an important role in stimulation of tumor cell growth, suppression of the immune response, induction of angiogenesis, and promotion of metastatic processes. Exosomes are derived from many types of biofluids; they function as stable carriers of cellular DNA, RNA, and proteins. There are several mechanisms that explain the formation of exosomes, including (i) formation of multivesicular bodies, (ii) direct budding at plasma membrane, and (iii) virus particle-mimic leaving from cell. Cancer cells have the capacity to produce more than 10^4 vesicles/day; ultimately, 1 ml of plasma may contain more than 10^8 vesicles. Exosome analysis easily detects tumor-specific mutations. Additionally, exosomes derived from tumor cells act as a shield containing genetic material and surface markers; therefore, they can be stored over an extended period. Taylor and Gercel-Taylor isolated circulating tumor exosomes using a modified MACS procedure with anti-EpCAM; then, they analyzed the microRNA profiles including miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214. The results showed that exosomal microRNAs from patients with ovarian cancer were significantly specific and similar to cellular microRNAs (Taylor and Gercel-Taylor, 2008).

Enumeration of circulating prostate microparticles (PMPs), a type of extracellular vesicle (EV), can identify and prioritize patients with different risk for prostate cancer (PCa) without assessing the levels of PSA. Biggs et al. used nanoscale flow cytometry to determine the levels of PMPs and compared them with CellSearch CTC subclasses in various subtypes of metastatic prostate cancer (PCa). The results showed that PMP levels in the plasma are far more effective than CTC subclasses in distinguishing PCa patients with different risks and prognostic factors. Moreover, PMP levels demonstrated the prognostic potential for clinical follow-up and could be used independently of PSA levels (Biggs et al., 2016).

Activated platelets contain numerous growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF β), insulin-like growth factor (IGF)-1, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). Cancer cells use a strategy of drawing, activating, and using growth factors secreted by platelets, turning platelets into tumor-educated blood platelets (TEPs). TEPs have been shown to alter their RNA profile, and using TEPs for mRNA sequencing

has diagnostic potential. Best et al. used platelet mRNA sequencing to distinguish patients with cancer from healthy individuals with extremely high accuracy. Additionally, TEP mRNA profiles could identify the location of primary tumors, as well as distinguish MET or HER2-positive and mutant KRAS, EGFR, or PIK3CA tumors (Best et al., 2015; Joosse and Pantel, 2015).

Li et al. isolated exosomes from various body fluids, sequenced the unique RNA cargo, labeled the exosomes, and presented the initial data in a cell culture model (Li et al., 2014). San Lucas et al. isolated exosomes shed in biofluids from patients with pancreaticobiliary cancers and performed comprehensive profiling of exoDNA and exoRNA by whole-genome sequencing using the Illumina HiSeq 2500 sequencer. The exoDNA sequencing data showed a robust presence of tumor DNA with multiple actionable mutations, including alterations in NOTCH1 and BRCA2, within the shed exosomal compartment. In exoRNA sequencing data, shed exosomes identified the presence of expressed fusion genes (San Lucas et al., 2016).

CONCLUSION

Advances in targeted cancer treatment have enhanced the ability to destroy cancer cells. However, the efficacy of cancer therapies is limited by rapid changes, which is a property of cancer cells. To overcome these obstacles, new treatment methods that can complement methods used for tumor management are recommended because “real-time” monitoring is paramount for effective therapy. Liquid biopsies, which go beyond the limitation of repeated cancer cell sampling needed to adjust therapy in response to tumor genetic changes, usher in a new era in the diagnosis and treatment of diverse cancers. Liquid biopsies contain many components, including CTCs, ctNAs (ctDNA, ctRNA), and exosomes. The analysis of each object has its own advantages and disadvantages; specifically, ctDNA analysis is appealing because ctDNA is simple to collect and analyze, but is limited to the analysis of DNA-related aberrations; in contrast, the analysis of CTCs provides profiling of the entire cell; however, it is difficult to enrich and isolate a population of rare cells. In obtaining the “whole picture in full colors”

characteristics of the tumor, it is clear that all the components of liquid biopsies have complementary roles as cancer biomarkers and hold great promise in the various facets of cancer management. Technological advances may allow high-throughput strategies for the assessment of clinical samples by ctDNA analysis, and functional studies may guide personalized treatment selection derived from the analysis of CTCs. Despite remarkable progress in identification using liquid biopsies, several challenges remain with respect to the question of whether blood-borne materials are specific to cancer cells. Understanding the details of how cancers spread will provide us with new treatment options at an early stage, which moves us towards personalized medicine. In order to establish clinical utility, we first need to optimize and standardize novel technologies for using liquid biopsies for analysis.

Competing Interests

The authors declare they have no competing interests.

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Cite this article as:

Vu, B., Le, D., & Pham, P. (2016). Liquid biopsies: tumor diagnosis and treatment monitoring. *Biomedical Research and Therapy*, 3(8): 745-756.