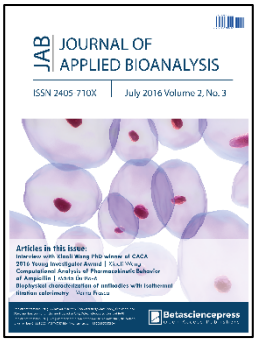

Clinical Applications of Circulating Tumor DNA, Circulating Tumor Cells, and Exosomes as Liquid Biopsy-Based Tumor Biomarkers

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Clinical Applications of Circulating Tumor DNA, Circulating Tumor Cells, and Exosomes as Liquid Biopsy-Based Tumor Biomarkers

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Abstract

Along with improved knowledge of cancer biology and biotechnical progress, the diagnostic approaches have evolved from tissue biopsies to liquid biopsies. As they provide a minimally invasive tumor detection, liquid biopsies allow early diagnosis and serial assessments of tumor progression.

Discovery and use of circulating tumor markers circulating tumor DNA (ctDNA), circulating tumor cells (CTC), and exosomes have largely expanded the possibility of early diagnosis of cancer, patient stratification, as well as developing a personalized treatment. Based on these circulomes, liquid biopsies can be developed, but each type of liquid biopsies has its own merits and limitations. While ctDNA-based methods represent the most advanced techniques, sensitivity improvement is expected given the rarity of ctDNA in circulation. As intact cancer cells, CTC provide information on cancer cells. However, current CTC capturing procedures are still lack of efficiency. Exosomes are abundant, but they are highly heterogeneous and there is a lack of specific markers for identification. Future efforts are needed to improve operational parameters and clinical performance of each method. Prior to a broad use in clinical settings, it is

crucial to standardize the procedure for the specific liquid biopsy method and validate the test with adequate specificity and sensitivity for clinical applications.

Key words: Immunotherapy, liquid biopsy, circulating tumor biomarker, cancer.

Introduction

Targeted therapies and immunotherapies have achieved a great success in treating cancer patients compared to conventional therapies such as chemotherapies. However, the treatment outcome largely depends on expression level of respective targets and immune checkpoints, which are usually assessed with a tissue biopsy. While tissue biopsies represent the gold standard for cancer diagnosis, frequent tissue biopsies are often impractical to perform due to invasiveness of the procedure and the risk of disease spreading it may incur [1, 2]. Moreover, the characteristics of cancer tissue can evolve during disease progression, the evidence acquired from tissue biopsies is, therefore, a limited snapshot of specific lesion at a specific time [3]. This limitation leads to incomplete information [4], possibly resulting in errors for tumor diagnosis and/or treatment decision [5].

In contrast to tissue biopsies, liquid biopsies usually use blood specimens that are much easier to collect and can be used to enrich blood-derived circulomes such as circulating tumor DNA (ctDNA), circulating tumor cells (CTC), and exosomes. These circulomes have been found to be related to tumorigenesis and thereby they could be important tools for tumor identification and treatment follow-up. ctDNA is part of cell free DNA (cfDNA). In 1989, cfDNA carrying neoplastic characteristics mutations was reported in plasma of cancer patients [6]. These circulating DNA are actually ctDNA. Since then, tumor-derived alterations, including specific gene mutations, epigenetic alterations, and copy number variations (CNV) have been broadly observed in ctDNA of cancer patients. Several commercial ctDNA detection kits have been approved for cancer diagnosis, such as cobas *EGFR* mutation test v2 (Roche) and Epi proColon (Epigenomics), both of which are elaborately discussed in ctDNA section [7-9]. Similarly, CTC are also shed by tumors and have been described in patients of several

cancer types, mainly breast, colorectal, and prostate cancers. CTC count of ≥ 5 CTC in 7.5 mL of blood has been demonstrated as a reliable prognostic tool for these cancers. CTC cluster was reported to have higher metastatic potential [10]. Now CTC detection has been incorporated into National Comprehensive Cancer Network (NCCN) clinical practice guideline (2017, v3) and American Joint Committee on Cancer (AJCC) staging manual (2018, 8th edition) for metastatic breast cancer diagnosis [11]. Unlike ctDNA and CTC, exosomes are abundant in biological fluids. They also closely correlate with tumors and are elevated in cancer patients compared to healthy controls. Moreover, exosomal proteins are significantly higher in cancer patients at advanced stages than those at early stages. The correlation between exosomal proteins and clinical outcomes could serve as predictive and prognostic biomarkers as protein levels of exosomes changed during and after chemotherapy [12]. Additionally, tumor derived exosomes (TDE) are able to induce oncogenic transformation of normal cells [13]. Thus, ctDNA, CTC and exosomes could serve as potential biomarkers for various clinical applications. This review focuses on the isolation procedures and the clinical applications of these circulating biomarkers for cancer diagnosis, tumor progression monitoring, patient selection, and evaluation of treatment effectiveness.

1. ctDNA

Cancer cells have long been known for harboring mutations of genes essential for cell growth control. For example, it was reported that in breast and colon cancers, almost every cancer cell contains approximately 80 mutated genes on average [14]. The ctDNA are tumor-derived DNA fragments found in blood. The typical length of ctDNA ranges from 80 to 200 bps and peaks at 160-180 bps, which is a bit shorter than majority of cfDNA [15]. While mechanisms of ctDNA formation are not fully understood,

apoptosis and necrosis of cancer cells are considered as major sources of ctDNA [16]. Active secretion of ctDNA by the cells in tumor tissue can be another mechanism [17].

The abundance of ctDNA was estimated to be less than 0.01% of total circulating DNA or cfDNA, equivalent to less than 10 ng/mL of plasma [18, 19]. Due to the rarity of ctDNA in blood, efficient enrichment and sensitive detection are necessary for many applications of ctDNA. A variety of nucleic acid isolation kits are commercially available for fast and convenient isolation of cfDNA. Two approaches, PCR-based and NGS-based methods, are primarily available for detection of gene mutations in ctDNA. While the former is usually used to detect small number of gene mutations, the latter is employed for detection of gene mutations of a large panel. Nowadays, with advent of sensitive digital polymerase chain reaction (PCR) technology, one can quantify as little as 0.001% ~ 0.01% mutated alleles in ctDNA samples [20]. In addition to these PCR-based approaches, a recently available technology named single molecular array (SIMOA) may provide another tool for ctDNA detection. It is based on direct detection of single molecules of DNA on magnetic beads. Briefly, ctDNA samples are subjected to denature to become single-strand DNAs, which are subsequently captured by magnetic beads through hybridization with specific probes (complementary sequences) attached to the beads. After hybridization with biotin-labeled probes, the complexes are incubated with streptavidin-β-galactosidase for detection. Although SIMOA technology is usually used for protein analysis, it is supersensitive in DNA detection with a reported limit of detection of 0.07 fM [21], providing an attractive alternative to the methods that rely on DNA amplification.

Along with discovery of tumor-driven gene mutations such as *EGFR*, *BRAF*, *KRAS*, and *P53*, ctDNA analysis has been extensively explored for cancer diagnosis and clinical response monitoring [22, 23]. It was reported that a panel of 16 genes, including

but not limited to *EGFR*, *BRAF*, *KRAS*, and *NRAS*, can be collectively used to detect eight common cancer types [24]. In a study of metastatic melanoma patients treated with immunotherapy, mutations of genes *BRAF*, *NRAS*, *TERT* and *ALK* were found in ctDNA samples from five of ten (50%) patients. Moreover, three patients with disease progression exhibited an increase in ctDNA levels [25]. In another study of breast cancer [26], ctDNA levels were reported to correlate with degree of tumor burden better than CA15-3, a typical protein biomarker used for monitoring breast cancer. ctDNA also displayed a better performance in monitoring the treatment response in this study. In addition to its potential applications in cancer diagnosis and treatment monitoring, ctDNA analysis of tumor-driven genes mutations can be used to predict drug resistance and cancer recurrence. For instance, dynamic ctDNA profiling of genes *TP53*, *PIK3CA*, *mTOR*, and *Pten* in HER2-positive breast cancer patients could be used to identify those resistant to anti-HER1/2 tyrosine kinase inhibitor therapy with sensitivity of 85.7% [27]. Additionally, simultaneous analysis of *KRAS*, *TP53*, *PIK3CA* and *APC* gene mutations in ctDNA samples from colorectal cancer patients could highly predict the recurrence of cancer within one year [28]. In colorectal cancer patients after surgical resection, postoperative gene mutations of *KRAS, APC and P53* detected in ctDNA samples indicated a high risk of recurrence, especially in patients without the adjuvant chemotherapy treatment (79% versus 9.8% at median follow-up of 27 months) [29]. All of these results indicated that ctDNA could serve as a promising biomarker for patient selection, treatment evaluation and recurrence monitoring, as well as drug resistance prediction. Clinical uses of ctDNA analyses were reported in various studies and some of them are summarized in Table 1.

Currently, there are two ctDNA-based tests approved by the Food and Drug Administration (FDA). The first one was approved in 2016 as a companion diagnostic

for detection of *EGFR* mutation in patients with non-small cell lung cancer (NSCLC) (cobas® EGFR Mutation Test v2, Roche). This test is to select the patients who would benefit from erlotinib and osimertinib treatment [7, 8]. It is based on an optimized quantitative PCR technology using pre-amplification to improve the detection efficiency of mutated alleles. The clinical specificity and sensitivity of this test in NSCLC were 97.9% and 72.1%, respectively. Compared with tissue biopsy-based assay, the cobas® *EGFR* Mutation Test v1, the concordance between ctDNA and tissue biopsy analyses was 91% [30]. Another approved diagnostic test is Epi proColon (Epigenomics). This test is used for colorectal cancer diagnosis and it is based on the detection of ctDNA methylation [31]. Tumor-specific epigenetics, including investigations into methylation and histone post-transcriptional modifications, represent important applications of ctDNA analysis [32]. In recent years, several studies have been conducted to analyze ctDNA methylation patterns for cancer screening, early diagnosis, and follow-up of the disease progression [33, 34]. Some examples in this application are shown in Table 1. Several NGS-based ctDNA tests have been marketed as laboratory-developed tests (LDT) since 2014, such as Guardant360® (Guardant Health, 2014), FoundationAct (Foundation Medicine, 2016), Oncotype SEQ (Genomic Health, 2016), and PlasmaSELECT (Personal Genome Diagnostics, 2015). They are used to detect four major types of gene alterations, including point mutation, insertions/deletions, copy number changes, and gene fusions. Normally offered in the Clinical Laboratory Improvement Amendments (CLIA)-regulated and College of American Pathology (CAP)-accredited laboratories, these tests have shown good performance. As an example, Guardant360® exhibits clinical sensitivity of 85% and specificity of 99.9% in advanced stage solid tumors, and the analytical sensitivity and specificity of this test were 100% and 99.99%, respectively [9]. For further information

about these LDT, readers can refer to review article [35].

While ctDNA analysis has shown a great potential in various clinical applications, it exhibits several challenges. First, it is difficult to obtain good quantity of ctDNA as its content in peripheral blood is extremely low (< 0.01% of 10 ng/mL plasma). Moreover, half-lives of ctDNA are reported to be 16 minutes to several hours [28], which are short and may partially explain low abundance of ctDNA. Second, ctDNA detection platforms can vary from genome-wide analysis to a single gene interrogation. These analyses exhibit different detection limits (0.1-1.0% for allele-specific PCR, 0.01-2.0% for NGS, and 0.01% for digital PCR and cancer personalized profiling by deep sequencing (CAPP-Seq)) [36]. Therefore, analysis results can vary with different platform technologies, rendering evaluation of results difficult among different laboratories that employ different platforms. Another challenge lies in poor concordance between liquid biopsies and tissue biopsies in certain cases. As an example, the concordance of EGFR L858R and *19del* mutation between ctDNA- and tissue biopsy-based analyses in NSCLC patients was 91%, while it was only 61% for T790M mutation [37]. This might be attributable to different disease stages, as high concordance between liquid and tissue biopsies of *PIK3CA* mutation status was observed in advanced breast cancer patients, but a poor concordance was reported in patients at early-stage [38].

Combined analyses of other biomarkers with ctDNA samples could be used to improve the clinical value of ctDNA-based liquid biopsies. An example was provided by Cohen et al, who reported that through a combined analysis of eight protein biomarkers and ctDNA markers, 8 different cancer types could be identified with specificity of > 99%, and sensitivity of 69% to 98%, depending on cancer type [24]. Additionally, a combined

analysis of DNA from both exosomes and CTC might be an alternative to obtain more reliable results in clinical applications [39].

2. CTC

CTC are tumor cells that detached from primary and metastatic tumor lesions and enter into blood circulation. Since their discovery in 1869, CTC have been widely identified in patients with breast cancer [40], non-small cell lung cancer [41], prostate cancer [42], colon cancer [43], and pancreatic cancer [44]. As intact cells derived from tumor, CTC can provide valuable information on tumor composition, heterogeneity, invasiveness, and drug resistance. As early as in 2004, it was found in a clinical study conducted in breast cancer patients that the number of CTC was inversely correlated to progression-free survival (PFS) and overall survival (OS) of the patients receiving an anti-cancer treatment [45]. Similar findings were also reported in patients with prostate cancer and patients with colorectal cancer (CRC) [46, 47]. In patients with castration-resistant prostate cancer, Danila et al reported that the 2-year OS was 46% in the patients with low level of CTC and 2% in the patients with high level of CTC, indicating a prognostic value of CTC [48]. More studies on the clinical applications of CTC enumeration are listed in Table 2.

In addition to CTC enumeration, genetic and protein analyses of CTC also offer a potential value for cancer diagnosis and prognosis. The commonly studied tumor-driven gene mutations were reported in CTC. For example, *EGFR* and *PIK3CA* mutations were observed in CTC of NSCLC and breast cancer patients, respectively [49, 50]. Moreover, the presence of *EGFR* mutation was found to be associated with shorter PFS in NSCLC patients and *PIK3CA* mutation were related to shorter OS in breast cancer patients. Further molecular analyses are summarized in Table 3. The studies demonstrated a significant value of CTC in cancer diagnosis, prognosis and

treatment response evaluation. Indeed, in patients with different cancers, CTC showed distinct pattern of CNV, which was consistent with results of tissue biopsy analysis from metastatic lesions in the same patients [51]. Similar results were also observed for DNA methylation in CTC. As reported by Chimonidou et al [52], the methylation patterns of tumor suppressor and metastasis suppressor genes in CTC were found remarkably distinct in patients versus healthy individuals, and in operable patients versus metastatic patients. All of these findings indicated that CTC could serve not only as a diagnostic biomarker, but also as a prognostic biomarker as they provided valuable information for tracking cancer metastasis. Protein analysis can be performed on CTC in clinical studies as well. A study conducted in patients with castration-resistance prostate cancer revealed androgen receptor variant 7 (Arv7)-positive CTC as specific predictive biomarker for prostate cancer [53]. In another study, expression of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) was analyzed in CTC [54]. It was found that these two proteins could represent different status of androgen receptor (AR) activation. Moreover, the elevated number of "AR-on" CTC (CTC with $PSA^{+}/PSMA^{-}$ phenotype) was associated with reduction in OS in patients. Recently, the expression of immune checkpoint inhibitors (e.g. PD-L1) and other therapeutic targets such as HER2 was also found in CTC [55]. The expression analysis of these therapeutic targets in CTC showed important clinical value in responsiveness and drug resistance evaluation. As an example, a study in NSCLC patients reported that the number of CTC with higher PD-L1 inversely correlated to clinical response. In other words, the more CTC expressing high level of PD-L1 in the patients, the stronger resistance to anti-PD1 antibody treatment [55].

The prevalence of CTC is approximately 5 to 200 CTC in 7.5 mL of blood. Given this rarity, CTC enrichment is critical for its clinical application. There are three strategies

employed for CTC enrichment, including immuno-affinity-based capture, isolation by size of epithelial tumor cells (ISET) [56], and microfluidics-based methods [57]. Immunoaffinity-based capture approaches are most widely used, and microfluidics-based methods are usually used in combination with immunoaffinity-based capture [58]. The comparison of these enrichment methods is summarized in Table 4. The interested readers could also refer to several reviews for more information [59, 60]. The most widely used platform for CTC enrichment is an epithelial adhesion molecule (EpCAM)-based immunoaffinity capture assay such as CellSearch® (Veridex). EpCAM is an epithelial cell-specific marker ubiquitously expressed on normal epithelial cells and cancer cells, but not on blood cells, whereas CD45 is the leukocyte-specific marker. Cytokeratins (CK) are also epithelial cell-specific markers and highly expressed on cancer cells. Thus, CTC are defined as $CD45^{-}CK^{+}EpCAM^{+}$ cells by immunofluorence staining. The cut-off threshold in this method for distinguishing positive from negative results is 5 CTC in 7.5 mL of blood. This method was approved by FDA in 2004 for prognosis of metastatic breast cancer [45]. Until now, it is the only FDA-cleared method for CTC enrichment.

Although the use of CellSearch® has resulted in remarkable progress in CTC detection, this method has inherent drawbacks as it only relies on the expression of EpCAM for CTC enrichment. EpCAM is not a unique biomarker for CTC, and it could be present on normal epithelia cells. On the other hand, a significant number of CTC lacks EpCAM expression in patients with a non-epithelial cancer, such as melanoma [61]. With cumulative knowledge on epithelial-mesenchymal transition (EMT), it becomes clear that mesenchymal CTC would express mesenchymal markers such as Vimentin and Twist [62-64], but not epithelial marker EpCAM. In addition, EpCAM on epithelial CTC could be down-regulated or even lost during CTC circulation [65]. The use of

EpCAM for CTC isolation would thereby miss out on these EpCAM-negative CTC. Recently, several subpopulations of CTC have been identified, which include epithelial CTC (E-CTC) that express EpCAM, mesenchymal CTC (M-CTC) that express vimentin, and biphenotypic E/M-CTC (expressing both EpCAM and vimentin), as well as circulating tumor microemboli (CTMs) [66]. Thus, the use of CellSearch® has resulted in low sensitivity (5-30%) of CTC detection [67], as it detects epithelial CTC only. These findings indicate that CTC in peripheral blood are highly heterogeneous, and thereby it is inadequate to identify and characterize CTC based on EpCAM marker only.

Despite encouraging advancements have been achieved in the last decades, several barriers still exist, which prevent wide-spread clinical applications of CTC. First, the rarity of CTC poses a significant challenge to CTC utilization, warranting enrichment of CTC with high purity and quality. Second, CTC are highly heterogeneous and have no universal markers. The identification of $CK^+EpCAM^+CD45^-$ cells as CTC is imprecise as quite a few CTC neither express CK nor EpCAM. Finally, CTC numeration is strongly dependent on the blood volume, enrichment method, and photographic or image processing system [68]. Distinct cutoffs were reported among different methods for distinguishing positive from negative samples (5 CTC/7.5 mL for CellSearch®, 50 CTC/mL for ISET, versus 14 CTC/mL for CTC-chip) [69, 70], rendering analytical results from different methods difficult to compare. Thus, the future efforts will be needed to improve current methodologies for expanded use of CTC in the following aspects: 1) Development of more useful isolation method that may combine different isolation mechanisms (e.g. Immunoaffinity-based capture and microfluidics); 2) Establishment of standard isolation method that yields as many CTC as possible without losing their heterogeneity; 3) Identification of more specific

markers to CTC, and with more defined criteria to objectively identify CTC; 4) Confirmation of clinical value of CTC as a diagnostic, prognostic or predictive biomarker through large-scale clinical trials. Advances in these aspects would enable the better value of CTC as biomarkers for tumor treatment management.

3. Exosomes

Exosomes are small lipid bilayer vesicles of 30 to 200 nanometers in diameter [71]. Released from almost every type of eukaryocytes through a sequential process, they are enriched with multiple cargoes of cellular origin, including lipids, proteins, and nucleic acids. Since the initial discovery in sheep reticulocytes in 1983 [72], exosomes have been considered as powerful shuttles for transporting biofunctional cargoes among cells, and have been implicated in both physiological and pathological processes. Studies have indicated that tumor-derived exosomes (TDE) participate in cancer development. As reported by Roccaro et al, the exosomes released from bone marrow-derived mesenchymal cells promoted the multiple myeloma (MM) development in animal models [73]. Similar result was observed in breast cancer cells that TDE could induce oncogenic transformation of normal cells [74]. This could be attributable to the involvement of exosomes in various processes that facilitated tumor progression, for example, angiogenesis, EMT, and drug resistance. Angiogenesis is possibly mediated by activation of the PAR2 signaling, an established angiogenic pathway, by exosomes [75]. The EMT regulators such as TGF-□, □-catenin, and tumor necrosis factor alpha (TNF□) are widely found in exosomes and they enhance migratory and invasive capacity of cancer cells [76]. Drug resistance is mediated by TDE possibly via several mechanisms, such as encapsulating and exporting the drugs from cancer cells [77], transferring multi-drug resistance (MDR)-associated proteins into cancer cells, and binding to the drugs and thereby blocking them [78].

Due to easy access from biological fluids and given their roles in tumor progression and drug resistance, exosomes have been considered as attractive tools for cancer diagnosis and treatment evaluation. A markedly increased release of exosomes was observed in the serum of lung adenocarcinoma patients compared to non-cancer subjects (2.85 mg/mL versus 0.77 mg/mL) [79, 80]. Both exosomal proteins and nucleic acids have been explored as biomarkers for clinical use. In a study of pancreatic adenocarcinoma, author revealed that the quantities of glypican-1-positive exosomes could be used to reliably distinguish the adenocarcinoma patients from non-cancer patients and healthy subjects [81]. The messenger RNA of *EGFRv III*, a common tumorigenic mutation known as an in-frame deletion of exons 2–7 in the coding sequence of *EGFR*, was found in the exosomes of glioblastoma patients [82].

There are many other cargoes reported in exosomes, which are listed in Table 5. One type of cargoes of exosomes is microRNA (miRNA). Contents of miRNA can be different in exosomes from cancer patients versus healthy subjects. In the aforementioned study of lung adenocarcinoma, the mean miRNA concentration was significantly higher in patients than in control subjects (158.6 ng/mL versus 68.1 ng/mL) [79]. Besides, it was found that various specific miRNA could discriminate cancer patients from healthy subjects. For example, 8 species of miRNA were uniformly elevated in the patients with advanced-stage ovarian tumor, while they remained at much lower levels in benign patients and even absent in healthy controls [83]. Recently, PD-L1 was found to be specifically packaged into exosomes of prostate cancer cells and to be involved in inhibiting T cell activation and promoting cancer cell growth [84]. Moreover, another clinical study of melanoma showed that exosomal PD-L1 was exclusively related to pembrozumab resistance, and the level of exosomal PD-L1 is much higher in non-responders than that in responders [85]. This finding

suggested that exosomal checkpoint inhibitor levels could serve as a potentially useful surrogate for predicting the clinical response in immunotherapy.

In summary, exosomes are multifunctional entities that play important roles in tumor progression and drug resistance. However, high quality exosomes are difficult to prepare as they are highly heterogeneous in terms of size, cargo content, and cellular origin. Five types of methods have been developed, which include differential centrifugation, size exclusion chromatography, immune-capture, polyethylene glycol precipitation, and microfluidic-based methods. These methods are based on the distinct features of density, size, surface proteins, and hydrophobicity of exosomes, respectively. The principles, applications and commercial products, as well as advantages and disadvantages of each method are provided in Table 6, and in a review article [86]. Despite availability of various methods for exosomes isolation, there is no single perfect method, and therefore methods are usually used in combination. As different laboratories use different methods and/or different markers for cancer identification, the results are hardly comparable among laboratories. Thus, further efforts are needed to standardize the exosomes-isolating method and to identify common markers for use.

Comparison of three circulating tumor markers

Circulating tumor markers ctDNA, CTC, and exosomes have largely expanded the possibility of early diagnosis of cancer, patient stratification, as well as developing a personalized treatment. Based on these circulomes liquid biopsies can be developed, but each type of liquid biopsies has its own merits and limitations. Among the three circulomes, ctDNA-based methods represent the most advanced techniques. Several commercial products or LDT platforms has been launched. As it detects gene mutations

with the sensitivity of 0.01%, it is broadly used for patient selection, medication guidance, and recurrence monitoring. However, it is difficult to distinguish real CNV from an operational error or aging-associated clonal hematopoietic mutations of indeterminate potential [87]. Moreover, PCR-based ctDNA detection is of low throughput and a sequencing-based method is of high cost. CTC is also an approved biomarker for prognosis of breast cancer. By enumeration, CTC is approved as adjunctive diagnosis for breast cancer staging. As intact cancer cells, they can be subjected to single cell analysis at both nucleic acid and protein levels. Furthermore, CTC can be cultivated in vitro for other analyses. However, CTC capture and analysis are costly. In addition, ambiguous correlation between CTC and tumor progression and treatment outcome might complicate cancer diagnosis and prognosis [88]. Exosomes are abundant, which makes them easier to obtain. Moreover, extensive studies have revealed that the miRNA enclosed in exosomes are differentially present in cancer patients versus healthy controls, which holds a great potential for cancer diagnosis. However, exosomes are highly heterogeneous and there is a lack of specific markers for identification. Thus, technologies remain to be further developed before one can effectively apply exosomes in cancer diagnosis. For further comparison among these three types of circulomes, readers can refer to Table 7.

Summary and future perspectives

These circulomes and the associated liquid biopsy methods elaborated above provide promising supplemental tools, and in some cases, the tool alternative to tissue biopsies. Although remarkable advances have been made in last few decades, clinical applications of the liquid biopsies is still challenging due to the rarity and difficulty in enrichment of these circulomes. Future efforts are needed to improve clinical performance of each method. For ctDNA analysis, efforts should be directed to develop

high throughput digital PCR instrument with a further improved signal-noise ratio. For CTC analysis, an urgent need would be to develop an immune-affinity enrichment protocol based on multiple antibodies against both epithelial and mesenchymal markers. For exosomes, the focus of efforts should be to identify cancer tissue origin of exosomes and to validate the correlation between exosomes and cancers in large clinical studies. It should be noted that except approved methods, the isolation procedures of circulomes and design of clinical trials vary significantly in different laboratories and clinical centers, resulting in poor comparability. Thus, prior to a broad use in clinical settings, it is crucial to standardize the procedure for the specific liquid biopsy method and validate the test with adequate specificity and sensitivity for clinical applications. Progresses in liquid biopsy technology would yield significant benefit for cancer diagnosis, patient selection, and treatment effect monitoring.

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Table 1. Summary of studies based on ctDNA analyses in cancer patients

Year	Type of cancer	Number of subjects	Enrichment method	Analytical method	Observations	Refs
2008	CRC	Patients: 133 healthy control: 179	MagNA Pure LC system (Roche)	qPCR and methylation analysis	The methylation of *TMEFF2*, *NGFR* and *SEPT9* in CRC patients were significantly different from healthy subjects	[88]
2016	NSCLC with acquired TKI resistance	117	QIAamp Circulating Nucleic Acid kit	ddPCR	T790M mutation ctDNA was identified 2.2 month earlier than clinically progressive disease. Moreover, the OS of the T790M ctDNA positive patients was significantly shorter than those negative patients.	[89]
2016	Metastatic CRC	97	Qiagen Blood Mini Kit	qPCR	Patients with high cfDNA levels or mutant ctDNA levels had significantly shorter OS. Moreover, the fragmentation of cfDNA positively correlated to the decreased OS in the patients with *KRAS/BRAF* mutation.	[26]
2019	Broad range of early phase cancer	100	Qiagen CAN manual kit and QIASymphony automated system	NGS (641 cancer-associated gene panel)	The PR of in the patients received matched therapy was 4/11, and 7/11 showed stable disease while the PR in the patients who received non-matched therapy was 0/17 and 4/17.	[90]
2020	NSCLC	65	QIAamp Circulating Nucleic Acid kit	CAPP-Seq	Patients with decreased ctDNA concentrations after consolidation therapy of ICI showed superior outcome than patients with rising ctDNA levels.	[53]

CAPP-Seq: cancer personalized profiling by deep sequencing; CRC: colorectal cancer; ddPCR: digital droplet polymerase chain reaction; ICI: immune checkpoint inhibition; NGS: next generation sequencing; NSCLC: non-small cell lung cancer; OS: overall survival; PR: partial response; qPCR: quantitative polymerase chain reaction

Table 2. Summary of studies based on CTC analysis in cancer patients

Year	Type of cancer	Number of subjects	Enrichment method	Detection markers	Positive Cutoff	Observations	Refs
2004	BC	177	CellSearch®	CK, CD45	≥ 5 CTC/7.5 mL Blood	The PFS in the patients with >5 CTC/7.5 mL Blood and patients with <5 CTC/7.5 mL Blood was 2.7 months *vs* 7.0 months; this clinical research led to the approval of CellSearch®	[44]
2012	BC	517	CellSearch®	CK, CD45	≥ 5 CTC/7.5 mL Blood	The presence of CTC strongly predicted the survival in all metastatic BC except for $HER2^+$ patients	[91]
2012	Metastatic BC, CRC, PC	breast cancer: 177, colorectal cancer: 428 prostate cancer:231	CellSearch®	CK, CD45	≥ 1 CTC/7.5 mL Blood	The frequency of CTC was proportional to the survival and the OS of the patients was reduced by 6.6 months for each 10-fold CTC increase	[92]
2015	CRC	287	CellSearch®	CK, CD45	≥ 1,2,3 CTC/7.5 mL blood	The presence of CTC was significantly correlated with worse OS and PFS in both of non-metastatic and metastatic groups	[93]
2016	GC	138 patients	CellSearch®	CK, CD45	≥ 3 CTC	ORR and DCR in the group with unfavorable post-therapy CTC numbers were much lower than those with favorable post-therapy CTC numbers; similar results were observed for OS and PFS after therapy	[94]

Year	Type of cancer	Number of subjects	Enrichment method	Detection markers	Positive Cutoff	Observations	Refs
2018	HNC and NSCLC	HNC: 23 NSCLC: 33	ClearCell FX system	CK, CD45	≥ 1 CTC/7.5 mL	CTC-positive HNC patients showed shorter PFS and PD-L1-positive CTC were significantly associated with worse outcome. However, there was no correlation between CTC and PFS in patients with NSCLC	[95]
2019	Lung cancer	123 patients and 59 control subjects	Negative enrichment	CD45, CEP8 (FISH)	≥ 2 CTC/3.2 mL	The diagnostic sensitivity of CTC and the combination of CTC and serum biomarkers in early-stage lung cancer patients (I-II) were 68.29% and 78.69%, respectively	[96]

BC: breast cancer; CK: cytokeratins; CRC: colorectal cancer; DCR: disease control rate; FISH: fluorescence in situ hybridization; GC: gastric cancer; HNC: head and neck cancer; NSCLC: non-small cell lung cancer; ORR : overall survival; PC: prostate cancer; PFS: progression free survival; OS: overal survival

Table 3. Genetic and proteinic analyses of CTC used as prognostic biomarker for cancer treatment evaluation

	Target	Cancer type	Observations	Refs
Genetic analysis	*EGFR* mutation (c.2369C>T)	NSCLC	The presence of EGFR mutation in CTC was correlated with a shorter PFS	[48]
	PIK3CA mutation	BC	The OS of patients with PIK3CA mutation on CTC was shorter than those without; but no correlation was found between PFS and PIK3CA mutation on CTC.	[49]
	19 gene Score	Melanoma	The patients with reduced CTC scores showed an improved PFS than those with increased CTC scores	[97]
	Copy number variation	Lung cancer	CNV was specific to cancer subtypes and might be used as diagnostic markers	[50]
	Copy number variation	Colon cancer	CNV in primary tumors showed accumulatively converging towards CNV in CTC	[98]
	DNA methylation	BC	The methylation of specific loci in CTC was related to CTC clustering and promoted cancer metastasis	[99]
	DNA methylation	BC	DNA methylation of tumor suppressor and metastasis suppressor genes were significantly different among advanced patients, operable patients, and healthy controls	[51]
Proteinic analysis	Arv7	Castration-resistance PC	Arv7-positive CTC were considered as a specific biomarker for evaluation of treatment outcomes	[52]
	PSA and PSMA	PC	The expression of PSA and PSMA in CTC could represent the status of estrogen receptor activation, and reflect the response of hormone therapy	[100]
	HER2	BC	CTC-HER2-postive patients showed longer PFS than CTC-HER2-negative patients, but no difference was observed between these patients for OS	[101]

	Target	Cancer type	Observations	Refs
Immune checkpoint analysis	PAR2	Glioma	Exosomes positive for PAR2 mediate the hypoxia-induced angiogenesis.	[74]
	PD-L1	NSCLC	the number of CTC with higher PD-L1 in patients showed negative correlation to the clinical response	[54]

Arv7: androgen receptor variant 7; BC: breast cancer; CNV: copy number variation; HNC: head and neck cancer; NSCLC: non-small cell lung cancer; PC: prostate cancer; PSA: Prostate-specific antigen; PSMA: prostate-specific membrane antigen; PAR2: proteinase-activated receptor-2; NSCLC: non-small cell lung cancer;

Table 4. List of methods used for CTC enrichment

Methods	Immunoaffinity capture		ISET	Microfluidic Methods
	EpCAM positive enrichment	CD45 negative enrichment		
Principle	Using EpCAM$^+$ magnetic beads to directly isolate the EpCAM$^+$ cells	Use CD45$^+$ magnetic beads to deplet the CD45$^+$ leukocytes	Cancer cells are usually larger than blood cells (8-20 μm versus 6-10 μm). The whole blood is filtered through 8 μm membrane	Based on different cellular size and deformability between cancer cells and blood cells, now it's usually combined with immunoaffinity
Commercial Product	CellSearch®; AdnaTest; OncoCEE; MagSweeper	EasySep Depletion Kit	ScreenCell; ISET	CTC-iChip; IsoFlux; Liquid Biopsy
Advantages	1. Low background 2. Easy to automation	1. Maintain the CTC heterogeneity 2. Easy to perform 3. Easy to automation	1. Easy to perform 2. Low cost 3. Retain the heterogeneity	1. Low background 2. Maintain the CTC heterogeneity 3. Can combine with other methods
Disadvantages	Lost EpCAM$^-$ CTC	High level of residual leukocyte	1. The membrane is prone to clogging 2. Lost small CTC and high background	1. High cost 2. Need special equipment

EpCAM: epithelial adhesion molecule

Table 5. Exosome cargoes used as biomarker in cancers diagnosis and prognosis

Cargo	Type of Cargo	Type of cancer	Observations	Refs
CD63	Protein	Melanoma	Significantly higher CD63 was found in cancer patients than in non-cancer patients	[102]
Glypican-1	Protein	Pancreatic adenocarcinoma	Glypican-1 could well distinguish pancreatic adenocarcinoma patients from non-cancer patients and healthy subjects. Moreover, the greater decrease of glypican-1 positive exosomes predicted longer OS in pancreatic cancer patients (26.2 months versus 15.5 months)	[80]
Survivin	Protein	Prostate cancer	Exosomal Survivin can be used for early detection	[103]
XIAP, cIAP1, cIAP2	Protein	Prostate cancer	May predict the aggressiveness of prostate cancer as these apoptosis inhibitors were significantly higher in the serum of African-American patients at more aggressive stage	[104, 105]
8 proteins including resistin and retinoic acid-induced protein 3,	Protein	Bladder cancer	These proteins were found differentially expressed in bladder cancer patients versus healthy control subjects	[106]
TACSTD,	Protein	Bladder cancer	urinary exosomal proteins	[107]
PCA3, and TMPRSS2-ERG fusion mutation	mRNA	Prostate cancer	Present in the urine exosomal	[108]

Cargo	Type of Cargo	Type of cancer	Observations	Refs
miR-21	miRNA	Breast cancer Lung cancer	Exosomal miR-21 was elevated in both lung cancer and breast cancer patients	[78, 109]
8 miRNAs including miR-21, miR-141 *et. al.*	miRNA	Ovarian cancer	The high level of these miRNAs could discriminate patients at advanced stage form benign subjects	[82]
8 miRNAs	miRNA	NSCLC	A panel of 8 miRNAs were found deregulated in clinical NSCLC plasma but not in normal subjects	[110]
miR-141	miRNA	Prostate cancer	Can discriminate the cancer patients from those with benign hyperplasia and healthy subjects with the specificity of 87.1% and sensitivity of 80%	[94]
miR-19a	miRNA	CRC	Correlated with recurrence of colorectal cancer, but also inversely associated with the OS of colorectal cancer patients	[111, 112]

cIAP: cellular inhibitor of apoptosis protein; CRC: colorectal cancer; ERG: ETS transcription factor; NSCLC: non-small cell lung cancer; OS: overall survival; PCA3: prostate cancer associated 3; TACSTD2: tumor-associated calcium-signal transducer 2; TMPRSS2: trans-membrane serine protease 2; XIAP: X-linked inhibitor of apoptosis protein

Table 6. List of methods for exosomes isolation

Methods	DC	SEC	Immunity-capture	PEG Precipitation
Principle	The density of exosomes is different from that of other extracellular vehicles	Exosomes are smaller than other extracellular vehicles	Use the antibodies targeting to exosomal proteins such as CD9, CD63, and CD81	Alter the water-excluding polymers to alter the solubility or dispersibility of exosomes
Application	Clinical therapy	Research	Research	Research
Commercial products	**N/A**	**N/A**	MagCapture Dynabeads Exosome-Streptavidin Latex immunobeads	Exoquick Total Exosome Isolation Kit MiRCURY Exosome isolation kit
Advantages	1. Maintain the heterogeneity 2. Obtain concentrated exosomes 3. No contamination of reagent 4. Scalable	1. Fast and inexpensive, and scalable 2. No co-isolation of protein aggregates 3. Maintain the heterogeneity	1. Simple and convenient 2. Obtain pure exosomes 3. Easy to combine with other methods	1. Fast and simple 2. Scalable 3. Concentrated product
Disadvantages	1. Laborious, time-consuming and low throughput 2. Cause protein aggregates	1. Low purity 2. Large elution volume	1. Low yield with subpopulation of marker-positive exosomes 2. Non-scalable 3. Large elution volume	1. Low purity 2. Low integrity 3. Contamination of organic reagent

DC: dencitycentrifuge; N/A: not applicable; PEG: polyethylene glycol; SEC: size-exclution chromotography

Table 7. Comparison of ctDNA, CTC and exosomes

	ctDNA	CTC	Exosomes
Abundance	< 100 pg/mL plasma	2-200 CTC/7.5 mL blood	0.88×10^8 to 13.38×10^8 exosomes/mL serum or plasma [88]
Half-life	16 minutes to several hours	1-2 hours	2 minutes to 6 hours in mice [89, 90], and no report in human.
Sensitivity of analytical method	0.01%	1 CTC	N/A
Types of detection	CNVs, point mutation, methylation	Change of CTC numbers Single cell sequencing	The presence and abundance of specific miRNA or proteins;
Analytical difficulties	1. Low abundance of mutations of interest that co-exist with much more abundant wild-type DNAs. 2. PCR-based methods are only practical for detection of known mutations	1. CTC are heterogeneous and extremely rare. 2. CTC enumeration is the only measurement for detection but it strongly dependent on the isolation and detection technique used. 3. The only approved platform is not specific and other methods are not standardized.	1. Inefficient separation methods 2. Difficulties in characterization 3. Lack of specific biomarkers 4. Heterogeneous and dynamically changed along with the physical/pathological conditions.

	ctDNA	CTC	Exosomes
Primary clinical application	Predictive and prognostic biomarker for cancers, as well as recurrence monitoring	Prognostic biomarker for BC, CRC, PC Recurrence monitoring	1. Primarily on its role as drug delivery system 2. Still under evaluation as liquid biomarkers and need further clinical validation
Advantages	1. High specificity and sensitivity 2. Well-established methods for isolation 3. Various detection methods 4. There are approved detection kits 5. Could detect the epigenetic alteration	1. Intact tumor cells which could be used for functional study 2. Allows single-cell sequencing 3. Allow protein expression evaluation 4. One platform has been approved	1. High abundance and are easy to obtain 2. Various cargoes of cellular origin 3. Contain stable miRNAs and proteins 4. Easy to identify 5. Could detect the miRNA signature alteration
Disadvantages	1. Extremely low; 2. Short half-life period; 3. Low signal-to-noise;	1. Low abundance in blood and are heterogeneous; 2. Difficult to isolate; 3. No specific markers for identification	1. Heterogeneous; 2. Difficult to isolate pure exosomes; 3. No approved platforms

BC: breast cancer; CNV: copy number variation; CRC: colorectal cancer; miRNA: microRNA; N/A: not applicable; PC: prostate cancer