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Review Article

Liquid Biopsy: Advances, Limitations and Clinical Applications

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Abstract

As an important part of precision medicine, liquid biopsy attracts more and more attention of scholars and clinicians. It refers to the real-time monitoring of the dynamic alterations of tumor by detecting circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes in patients' plasma or serum. Thus, liquid biopsy has irreplaceable advantages in tumor early diagnosis, progression monitoring, curative effects evaluation, prognosis judgement and so on as a non-invasive method. Here we reviewed traditional clinical diagnostic technologies and the liquid biopsy and summarized the advantages, limitations and test indexes of CTCs, ctDNAs and exosomes for their clinical applications. We also enumerated other applications of liquid biopsy by using other body fluids to monitor some particular cancers. At last, we forecasted the opportunities and existed challenges of liquid biopsy.

ABBREVIATIONS

CEA: Carcinoembryonic Antigen; PSA: Prostatic Specific Antigen; NGS: Next Generation Sequencing; CTC: Circulating Tumor Cell; cfDNA: cell free DNA; ctDNA: circulating tumor DNA

INTRODUCTION

With high morbidity and mortality, cancer has become a big health threatproblem around the world [1,2]. There are two main techniques of traditional tumor clinical diagnostic: pathology and medical imaging. Pathological diagnosis technology mainly includes tissue biopsies, serological indicators (eg. CEA and PSA) test and molecular pathology test (eg. FISH, RT-PCR and NGS) [3]. On the other hand, medical imaging methods for clinical application include ultrasonic testing, X-ray imaging, CT, MRI, PET-CT and endoscope. However, there exist inevitable disadvantages of these approachs. For example, the result of imaging usually lags behind tumor progression for medical imaging approach. And it lacks effective biomarkers for early diagnosis in serological indicators test, thus need further clinical examinations. As for tissue biopsy, though recognized as the current gold standard for tumor diagnosis, it also has its disadvantages due to the severe clinical complications resulting from sampling and the result bias caused by tumor heterogeneity. The most important, sometimes it is very difficult to obtain tissues, especially from terminal cancer patients. Thus, it is necessary to find new biomarkers which have high specificity and sensitivity for tumor diagnosis and can be used to dynamically and timely monitor tumor progression.

Tumor tissue will release tumor cells, DNA and exosomes into the body fluid, which offers a test approach called "liquid biopsy" (Figure 1) [4]. It means diagnosis and monitoring tumor initiation and progression by capturing and detecting biomarkers (eg. cell, DNA, RNA and protein) in body fluid (eg. blood, urine and saliva). The greatest strength of liquid biopsy is to allow doctors to noninvasively take repeated tumor samples. Other advantages include fewer side effects, ease of operation, rapid testing speed, decreasing the diagnosis bias from tumor heterogeneity and dynamically reflecting tumor progression. With these advantages, liquid biopsy has a wonderful future in the field of early diagnosis, tumor drug design, elucidating drug-resistant mechanism, estimation of tumor's grade and stage, judging prognosis and guiding treatment plan.

DETECTION TARGETS AND METHODS OF LIQUID BIOPSY

In 1869, Ashworth first found tumor cells in blood released by solid tumor and named it as circulating tumor cell (CTC) (Figure 1) [5]. In 2002 and 2003, Thiery and Steeg found the formation mechanism of CTC: after the emergence of primary carcinoma lined by an intact basement membrane *in situ*, activation/suppression of several pathways in it could induce an epithelial-mesenchymal transition (EMT), which means the basement membrane was fragmented and the epithelial cells of solid tumors intravasated into blood circulation and became solitary endothelial cells. These endothelial cells, which were also

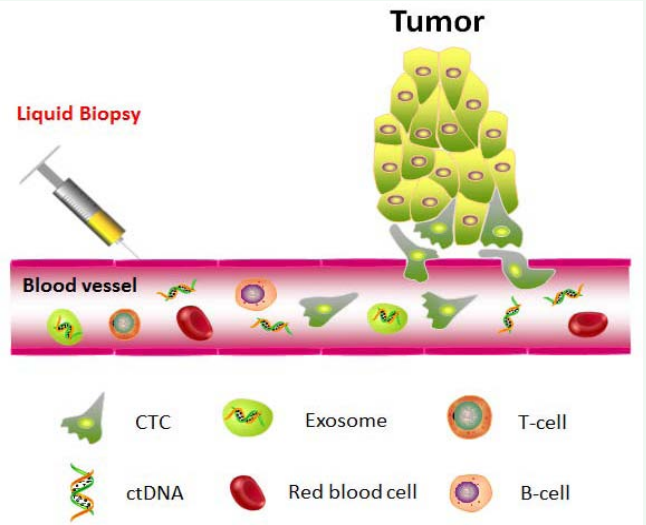

Figure 1 CTC, ctDNA and exosome in the blood. CTC, ctDNA and exosomecan be released in different forms and at different levels by tumor cells through apoptosis, necrosis and secretion. They can be detected in body fluid, such as blood, as biomarkers for real-time monitoring tumor initiation, progression, metastasis and drug-resistance. This noninvasively test approach is named as "liquid biopsy".

named as CTCs now, couldsurvive in the blood, be transported to distant organsand might lead to micrometastasisor form a new metastatic carcinoma by a reversemesenchymal-epithelial transition (MET) [6,7]. During EMT, the down-regulation of E-cadherin leads to the loss of epithelial phenotype, while the modulation of other adhesion systems and the remodelling of actin cytoskeleton lead to the establishment of mesenchymal phenotype [6]. Since CTC carries phenotypic and genotypic information of solid tumor, it has been recommended as new tumor biomarker by American Society of Clinical Oncology (ASCO) in 2007. Although the mechanisms of CTC's anoikis resistance and metastasis initiating potential are still unclear, it has become a novel hotspot in the therapy of many cancers such as breast, prostate, lung and colorectal cancer for years [8-12]. However, there are some limitations of CTC as a liquid biopsy biomarker. For instance, CTCs have a complex heterogeneity because of their original tumor cells' known heterogeneity. First, the morphology of CTCs derived from different tumor tissues through EMT is significantly different. Second, even within only one cancer, the morphology and amount of CTCs derived from different molecular subtypes of solid tumors or distant sites (eg. prostate primary and bone metastatic cancers) are distinct. Third, in clinical practice, the percentage of patients whose CTCs can be detected is also different between cancers (eg. colorectal, ovarian and breast cancer is 50-70%, while non-small cell lung cancer is only 30%). This inter- and intra-patient heterogeneity mentioned above might lead to diagnosis bias caused by missing some small subclones of tumor cells like tissue biopsy. Thus doctors should reach an agreement on a clinically minimum number of profiled CTCs to account for heterogeneity in the future. Besides, the phenotypic diversity of CTCs also presents a challenge for choosing appropriate detection assay conditions. Because of CTCs' low concentration, ranging from 1 to 10 cells per 10mL peripheral blood, it is necessary to enrich CTCs before analysis [13]. At present, there are three enrichment technology principles: biological properties (eg. immunoaffinity of antibody and cell surface antigen), physical properties (eg. CTCs' density, size and surface charge) and directly analyzing CTCs in the blood [13]. The enrichment technology using biological properties includes positive (eg. EPCAM and CellSearch® system) and negative selection [14-16]. Among them, the counts of CTCs detected by CellSearch® system has been approved to clinical use to judge prognosis of prostate, colorectal and metastatic breast cancer by FDA. So far, this is the only liquid biopsy technology approved by FDA. After enrichment, there are many methods for analysis of CTCs, such as immunophenotyping, FISH, target PCR, DNA sequencing, RT-PCR and RNA-seq [17]. As a part of CellSearch® system, immunophenotyping ($EpCAM^+CK^+DAPI^+CD45^-$) has become a gold standard of CTC detection [17,18].

In 1948, Mandel and Métais first found cell free DNA in health people's blood [19]. In 1977, Leon et al first found the concentration of this DNA raised in cancer patients' blood [20]. After that, more and more evidences proving that tumor cells could release fragmentary single/double strand DNA to peripheral blood by necroptosis, apoptosis and secretion were observed (Figure 1). Finally it was named as cell free DNA (cfDNA) [21]. The length of cfDNA ranges 70-200bp and the average concentration in cancer patients' blood is 180ng/mL [22]. Among cfDNA, those released by solid tumor were named as circulating tumor DNA (ctDNA). ctDNA has been detected in various cancer patients' blood and its mutations show a significantly positive correlation with tumor's stage and grade malignancy because they carry with the genotypic and methylated information of their original solid tumor [22]. Moreover, ctDNA's half-life is less than two hours, which means it can be used to monitor tumor progression timely. Thus ctDNA has become a typical detection target of real-time liquid biopsy. Currently, researchers usually use plasma instead of serum to extract cfDNA to avoid the interference of white blood cells' genome. However, the percentage of ctDNA in cfDNA is very low (0.01%-1%). So the detection of ctDNA becomes a challenge. Some existed ctDNA's genomic variations detection technologies include droplet digital PCR (ddPCR), BEAMing, ARMS, TAM-Seq and CAPP-Seq [23-26]. On the other hand, there are some methods developed for detection of ctDNA's methylation profile, such as methylation-specific PCR (MSP), qRT-MSP, cMethDNA array, methylation on beads (MOB), BS-Seq and methylated CpG tandems amplification sequencing (MCTA-seq) [27-31].

Exosomes are small (50–100nm in diameter) and lipid bilayer membrane vesicles of endocytic origin. Their density ranges 1.13-1.19g/mL [32]. They are released by various cells (eg. tumor, immune and nerve cells) and can move along with blood circulation (Figure 1) [32]. According to the database established specifically for exosomes (http://exocarta.org), researchers have found a wide variety of inclusions in exosomes, including 9769 proteins (eg. CD9, MHC-I, EGFR, TSG101 and Hsp70), 3408 mRNAs and 2838 miRNAs [32,33]. Many studies have proved that exosomes play roles in tumor initiation, progression, metastasis and drug-resistance [6,32-37]. Based on these results, researchers have developed some exosomes targeted drugs and tried to use exosomes for drug delivery [38-

40]. The enrichment methods of exosomes are similar to those of CTC. After enrichment, many regular biological technologies, such as electron microscope, RT-PCR, western blot, FISH, flow cytometry and NGS, are used for exosomes analysis [41,42].

CTC, ctDNA and exosomes are three typical detection targets of liquid biopsy. Among them, it is relatively far for exosomes from lab to clinical application because of lacking effective enrichment technologies and precise quantitative analysis methods (Table 1) [32]. Comparatively, CTC and ctDNA have more alluring prospect of clinical application. Both CTC and ctDNA have their own advantages and disadvantages and their results can supplement each other (Table 1). However, both of them have the problem of false positive and false negative in clinical diagnosis. Compared with CTC, the sensitivity and specificity of ctDNA's detection can significantly rise accompanied with the rapid development of NGS. Thus it might be widely applied in clinical test ahead of CTC. In fact, European Union has approved ctDNA test in the Iressa treatment of non-small cell lung cancer in 2014.

TEST INDEXES OF LIQUID BIOPSY

There are many different test indexes for CTC, ctDNA and exosomes. First, the amount of CTC can be used for tumor diagnosis. For instance, it suggests that the prognosis of prostate and metastatic breast cancer will be poor if 5 or more CTCs are detected out by CellSearch® system in 7.5mL peripheral blood. Second, tumor marker proteins on the surface or inside of CTC or exosomes can be tested as another kind of index, such as EpCAM, CK8, CK18, CK19, CD44, CD24, ALDH1 and Hsp72 [35,43-46]. Third, the integrity of ctDNA can also be detected as an index. Some studies have reported that the breast and ovarian cancer patients lacking ctDNA microsatellite heterozygosity have poor prognosis [47,48]. Fourth, many genes' mutation were found in CTC, ctDNA and exosomes, such as APC, EGFR, ESR1, PIK3CA and KRAS [23,49-55]. These genes' mutation frequencies were related to tumor grade, chemotherapy and drug-resistance and thus can be used to timely monitor tumor progression and curative effect. For example, the levels of APC G4189T mutation in colorectal cancer patients' ctDNA decreased after surgery and increased when recurrence was identified by radiological examination [53]. Thus it could be seen as a measure of tumor burden and used to monitor tumor progression and recurrence. Compared to APC, within the same cancer type, another studies reported that several mutations of KRAS in ctDNA almost did not exist before treatment but significantly emerged and increased during anti-EGFR therapy, following by the emergence of clinically resistance after several months [51,54]. Thus it could be used as a new molecular measure of acquired resistance to targeted agents. In the future clinical practice, multiple biomarkers including mutations of both APC and KRAS would be profiled to obtain a reliable global picture of colorectal cancer. Another important and popular subject is the mutations of EGFR in serum, which can affect EGFR blockade therapies and reflect the dynamic changes of tumor ahead of medical imaging for several months [26,55]. Fifth, DNA methylation profile is a very important test index for now. Pack et al., found SEPT9's hypermethylation in ctDNA is closely related to colorectal cancer progression [56]. Similarly, the methylation levels of APC, RASSFIA, DAP-kinase and KIF1A are significantly changed in breast cancer patients' ctDNA [57-59]. Especially, Bryzgunova et al. found the promoter of GSTP1 was hypermethylated in ctDNA from prostate cancer patients' blood and urine, which is consistent with the result of tissue biopsy [60,61]. Unlike mutations, DNA methylation is tissues/cell types-specific, which means we can figure out the origin of ctDNA by comparing its DNA methylation pattern to known organs' [62]. Finally, the up-regulation of some RNA, such as TTF-1 and ICAM-1 mRNAs, are also reported in cancer patients' peripheral blood [63,64].

CLINICAL APPLICATIONS OF LIQUID BIOPSY

As biomarkers that can timely and dynamically reflect tumor's status, CTC, ctDNA and exosomes have broad application prospects in many stages of tumor therapy. First, early diagnosis

Table 1: Advantages, limitations and test indexes of CTCs, ctDNAs and exosomes.

Subject	Advantages	Limitations	Test indexes
CTCs	Count is significantly correlation with prognosis, the currently only one liquid biopsy method approved by FDA Allow both phenotypic and genotypic analysis, including cell morphology, NGS, protein localization and other immunolabeling-based approaches Potential relation with the tumor progression and metastasis Various technologies for CTC enrichment or isolation High specificity Allow culturing and analysis *in vitro*	High heterogeneity of morphology and count in different cancers and patients Low abundance and fragility False negative and false positive results Cannot decrease the diagnosis bias from tumor heterogeneity	Count Maker proteins Mutation DNA methylation RNA
ctDNAs	High sensitivity Allow analysis of DNA sequence and methylation, including PCR and NGS Can decrease the diagnosis bias from tumor heterogeneity Timely and dynamically monitor tumor progression	Low specificity because of cfDNA from normal tissues False negative and false positive results	Concentration DNA integrity Microsatellite alterations Mutation DNA methylation
Exosomes	Allow analysis of DNA, RNA and proteins from solid tumor Potential relation with the tumor drug-resistance and metastasis	Lacking effective enrichment method	Concentration Maker proteins Mutation DNA methylation RNA

is one of the most important application area that liquid biopsy displays the most advantages than tissue biopsy and imaging examinations. Limited by minute size of lesions, tissue biopsy and imaging examinations have difficulty in finding early tumor. In contrast, biomarkers of many cancers can be detected in peripheral blood at an earlier stage, which can significantly improve the survival rate of patients by early detection and treatment [65]. Second, liquid biopsy can be used to monitoring tumor progression. Genes' mutations' frequencies in ctDNA and its dynamic alteration are positively correlated to tumor's grade, stage and evolution, which can assist doctors to estimate tumor progression and evolution [22,65,66]. Liquid biopsy can also be used to monitoring and predicting the curative effect and risk of recurrence. For example, Thress et al., found lung cancer patients with EGFR C797S mutation in ctDNA developed resistance to drug AZD9291, which suggested the waning curative effect of this targeted drugs and the need to switch to another medication [64-67]. In addition, PIK3CA mutations in ctDNA can be used to detect breast cancer's minimal residual disease (MRD) after chemotherapy or surgery [68]. At last, the DNA integrity, microsatellite alterations, mutation and methylation of ctDNA were all reported to relate to the prognosis of various cancers thus can be used to predicting the prognosis and helping doctors adopt the most suitable treatment to each one patient [22].

Besides peripheral blood, it is worth noting that other body fluids can also be used to monitor some particular cancers. For instance, the methylation pattern of a CpG site was identified as a biomarker for prostate cancer non-invasive diagnosis by urine DNA methylation detection [69]. Its sensitivity is 94.6% and specificity is 78.3%, which is better than PSA concentration (clinical index). In addition, Erbes et al., found that the difference in nine miRNAs' expression levels in urine could distinguished the breast cancer patients from other non-cancer ones (both sensitivity and specificity are 91.7%) [70]. Based on these researches, we can speculate that other methods, such as using saliva to test esophagus cancer and using urine to test renal or bladder cancer, might be developed in the future.

DISCUSSION & CONCLUSION

In summary, liquid biopsy have many advantages: noninvasively and repeatedly taking sample, timely and dynamically monitoring tumor initiation and progression, judging prognosis and assisting doctors to change treatment plan. However, there still exist some challenges that obstruct the development and application of liquid biopsy in clinical practice.

First, because of low concentration of CTC, ctDNA and exosomes, the sensitivity, specificity and robustness of current detection technologies need to be improved. In addition, it is difficult to detect gene mutations, amplifications and fusions in parallel in such a low concentration of samples. Compared with the most commonly used ddPCR, which can only detect gene mutations, NGS can detect all of indexes in parallel with the current highest (but still not high enough) sensitivity. Moreover, despite rapidly falling down of NGS's cost, it is still high for clinical practice. Second, the sample size in most of current researches of liquid biopsy is small (range 3-1000). It is necessary to test these studies' conclusion in more clinical samples and answer these key questions: Can the sensitivities and specificities of biomarkers screed out using a few samples keep high in thousands of patients? How to deal with a biomarker's different representations in different patients and different cancers? Can we find a type of biomarker which can detect various tumors at one time? For answering these questions and using liquid biopsy in clinical practice, these biomarkers need further tested. Third, the standardization of every step of liquid biopsy, including sampling, enrichment, NGS, gene annotation, analysis result interpreting and inspection reports issuing, is necessary but lacking. Finally, liquid biopsy must benefit patients, which means two things. On one hand, no matter early diagnosis, monitoring tumor progression or judging prognosis, the ultimate meaning of liquid biopsy is to improve cancer patients' lives, including overall survival, median survival time and progression-free survival. A previous study on metastatic breast cancer patients showed that despite close correlation of CTCs' counts and prognosis, the survival of patients with increased CTCs after chemotherapy was not improved after changing treatment plan [71]. On the other hand, there is no need to excessively pursue high sensitivity of detection technology. It has been reported that despite higher sensitivity of amplification refractory mutation system (ARMS) than NGS, non-small cell lung cancer patients treated by gefitinib could not benefit from it [72]. Thus, when people develop new detection technologies or find new biomarkers, whether patients benefit from it, rather than technologies or biomarkers themselves, is the ultimate criterion.

Although some problems still exist, there is no doubt that liquid biopsy is welcomed by doctors and markets because of its huge potential. With continuous improvement of detection technologies, more and more new found biomarkers and verification by large-scale clinical samples, we believe that liquid biopsy will become a strong supplement to traditional detection methods and play significant roles in tumor prevention, early diagnosis, therapy and pathogenesis research in the near future.

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